Acquired macrolide resistance genes in nontypeable *Haemophilus influenzae*

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Declaration of Originality

This thesis entitled "Acquired macrolide resistance genes in nontypeable *Haemophilus influenzae*", describes original research conducted by the candidate within the School of Health Sciences (formerly School of Human Life Sciences) at the University of Tasmania, and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information or duly acknowledged in the thesis, and to the best of my knowledge and belief no material has been published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The isolates used in the studies that comprise the thesis were from existing culture collections, acquired as part of routine care. As such, their use is exempt from ethical review according to the "National Statement of Ethical Conduct in Human Research" developed by the National Health and Medical Research Council, the Australian Research Council and the Australian Vice-Chancellor's Committee in 2007, because it is both a) negligible risk and b) involves existing collections of non-identifiable data. This exemption was confirmed by the Human Research Ethics Committee of the University of Tasmania.

The cloning work involving the acquired macrolide resistance genes described in Chapter 6 of this thesis was approved as an "Exempt Dealing" by the University of Tasmania Institutional Biosafety Committee (Ref 3-2014) in accordance with Schedule 2 of the Gene Technology Regulations 2001 and under the authority of the Office of the Gene Technology Regulator (Department of Health, Australian Government)

Candidate	Primary Supervisor
Christopher T. Atkinson	Dr. Stephen Tristram
Date 6th January 2017	Date 6th January 2017

Statement of Co-Authorship of Jointly Published Work

I was the primary author and lead investigator of the second published letter comprising Appendix 1 of this thesis. The letter is published as:

Atkinson CT, Kunde DA, Tristram SG. Acquired macrolide resistance genes in *Haemophilus influenzae*? – authors' response. *J Antimicrob Chemother* 2015; **70**: 3409-10.

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Statement of Candidature Contribution to Thesis

The thesis comprises interlinked research investigations where the candidate **Christopher Atkinson** is the lead investigator; however, the following people and institutions also contributed to the published and non-published work contained within the thesis as follows:

- Christopher Atkinson (School of Health Sciences, University of Tasmania): Lead investigator responsible for design of each individual research project, laboratory and experimental analysis, data collection, data analysis, data interpretation, and is the lead author on any result manuscripts.
- Dr. Stephen Tristram (School of Health Sciences, University of Tasmania): Assisted with research project design, experimental techniques and manuscript revisions.
- Dr. Dale Kunde (School of Health Sciences, University of Tasmania): Technical assistance in experimental work performed within the molecular biology laboratory and manuscript revisions.
- Bowen Zhang (School of Health Sciences, University of Tasmania): Assisted with the isolate identification (Chapter 4).
- Derek Sarovich (Menzies School of Health Research, Darwin, Australia): Assisted with experimental design and techniques (Chapter 5).
- Stefan Schwarz (Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Germany): Assisted with experimental design and techniques (Chapter 7).
- Geovana Brenner Michael (Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Germany): Assisted with experimental design and techniques (Chapter 7).

Special Contributions to Chapters

Chapter 4: Christopher Atkinson (70%), Stephen Tristram (15%), Dale Kunde (10%), Bowen Zhang (5%).

Chapter 5: Christopher Atkinson (70%), Stephen Tristram (15%), Dale Kunde (10%), Derek Sarovich (5%).

Chapter 6: Christopher Atkinson (80%), Stephen Tristram (15%), Dale Kunde (5%).

Chapter 7: Christopher Atkinson (60%), Stephen Tristram (10%), Geovana Brenner Michael (10%), Stefan Schwarz (10%), Dale Kunde (10%)

We, the undersigned, agree with the above stated "proportion of work undertaken" for each of the chapters which contributed to this thesis.

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Publications and Presentations at Conferences during PhD Candidature

A. Published Manuscripts

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Atkinson CT, Kunde DA, Tristram SG (2015). Acquired macrolide resistance genes in *Haemophilus influenzae*? – authors' response. *J Antimicrob Chemother*; 70 (12): 3409-10. [doi: 10.1093/jac/dkv290].

Atkinson CT, Tristram SG (2016). Antimicrobial resistance in cystic fibrosis isolates of *Haemophilus influenzae*. *Br J Biomed Sci*; 73 (2): 87-9. [doi: 10.1080/09674845.2016.1165408].

Atkinson CT (2016). BD student Awardee values networking opportunities offered by the ASM conference. *Microbiology Australia*; 37 (2): 98. [doi: 10.1071/MA16032].

B. Conference Presentations

Atkinson C, Tristram S, Kunde D (2014). Acquired macrolide resistance genes in *Haemophilus influenzae*. Proceedings from the 42nd Australian Society for Microbiology Annual Scientific Meeting and Trade Exhibition, 6th-9th July, Pullman Melbourne Albert Park, Victoria, Australia, Poster p208.

Atkinson C (2015). Acquired macrolide resistance genes and *Haemophilus influenzae*. Oral presentation proceedings from the 43rd Australian Society for Microbiology Annual Scientific Meeting and Trade Exhibition, 2nd-5th July, QT Canberra, Australian Capital Territory, Australia.

Atkinson C, Michael GB, Schwarz S, Tristram S (2016). Conjugal transfer of select acquired macrolide resistance genes from *Pasteurella multocida* to *Haemophilus influenzae*. Proceedings from the 44th Australian Society for Microbiology Annual Scientific Meeting and Trade Exhibition, 3rd-6th July, Perth Convention and Exhibition Centre, Western Australia, Australia, Poster p204.

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Thank you.

Yours Sincerely,

Christopher T. Atkinson

General Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that is associated with a range of respiratory infections, including acute exacerbations of chronic obstructive pulmonary disease (COPD), and community acquired pneumonia (CAP). Macrolide antibiotics such as azithromycin are being more frequently used to manage these conditions, including those where NTHi may be involved, despite macrolides having relatively poor antibiotic activity against *H. influenzae* (azithromycin MICs of wild-type strains typically cover the range of 0.25-4 µg/mL). The efficacy of these antibiotics in managing these conditions is further threatened by the emergence of strains of NTHi exhibiting high-level macrolide resistance.

A range of different mechanisms of macrolide resistance are recognised broadly within bacteria. Resistance has been attributed to the presence of mutations in structural components of the ribosomal binding site of macrolides. Such mutations can occur in the 23S rRNA gene, as well as in the L4 and L22 structural proteins. Increased expression of inherent macrolide efflux mechanisms such as *acrAB* resulting from mutations in regulatory regions of these efflux pumps have also been recognised as a potential cause of macrolide resistance. Finally, resistance has also been attributed to the acquisition of macrolide resistance genes (AMRGs), which are readily disseminated among species on mobile genetic elements. There are a large number of different AMRGs and associated proteins, and the mechanisms by which they produce resistance vary. For example, the *erm* genes encode enzymes which modify the ribosomal binding site, the *mef* genes encode alternate efflux systems, and an additional group of genes encode enzymes which directly deactivate the macrolide.

High-level macrolide resistance in NTHi is uncommon and has historically been attributed to chromosomal mutations in ribosomal structural elements or regulatory regions controlling *acrAB*. In contrast, AMRGs have not been widely associated with NTHi and a number of studies have failed to detect these genes in this particular species. There has been a single study in which these genes have been reported to be highly prevalent in NTHi. In that study, among a collection of 106 NTHi isolates from children with cystic fibrosis enrolled in a placebo-controlled azithromycin trial, all isolates had at least one AMRG, with many of the isolates carrying two or three of these genes. However, the phenotypic effect of these genes was not consistent; only 27 of the AMRG-carrying isolates exhibited phenotypic resistance to macrolides. While the findings of that study identify the emergence and potential spread of AMRGs in NTHi, it raises a number of questions regarding the prevalence of these genes within NTHi isolates more broadly and the role they play in generating a resistant phenotype.

In Chapter 4 of this thesis, a collection of 186 NTHi respiratory isolates of variable azithromycin resistance phenotype (azithromycin MIC range: 0.09 to >256 µg/mL; MIC50: 1.5 µg/mL; MIC90: 3 µg/mL) derived from both cystic fibrosis and non-cystic fibrosis patients was established and analysed for; 1) the presence of macrolide resistance-associated L4, L22 and 23S rRNA mutations, and 2) the presence of the AMRGs *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*(A) and *mef*(E). For the detection of the AMRGs, two methods were used; 1) a novel PCR using locked nucleic acid dual-labelled hydrolysis probes, and 2) the original primer set used by the authors of the previous study. While L22 and 23S rRNA alterations were detected in 2 isolates with high-level macrolide resistance (azithromycin MIC $\geq$ 256 µg/mL), none of the isolates were found to carry any of the AMRGs using the novel PCR detection method. When using the primers described in the original study, *mef*(A) and *erm*(A) were detected in a number of isolates. Subsequent analysis of these amplicons

revealed them to be false positive results, raising questions as to the possibility of false positive results in the original study.

Over 100 different AMRGs have been recognised, and the development and increasing availability of whole genome sequencing (WGS) techniques now allows for efficient and thorough analysis of sequences for the detection of antibiotic resistance mechanisms. In Chapter 5 of this thesis, WGS was utilised to further investigate the presence of a broad selection of other AMRGs (n=72) in NTHi, using the SPANDx pipeline. WGSs of two isolates of NTHi exhibiting high-level macrolide resistance obtained from the study of Chapter 4, as well as an additional 89 publically available WGSs of NTHi isolates of variable resistance phenotype, were examined in the study. None of the specified AMRGs were detected among this collection of WGSs. In addition, the WGSs of the 2 isolates from Chapter 4 with high-level macrolide resistance were interrogated for any AMRGs using the Comprehensive Antibiotic Resistance Database (CARD), and none were detected. Both isolates underwent further WGS analysis to confirm the L22 and 23S findings in Chapter 4; one isolate carried R88P in L22 and C2611G in 23S rRNA and the other isolate carried A2058G in 23S rRNA, all previously associated with decreased macrolide susceptibility in *H. influenzae*. Alterations in regulatory regions of *acrAB* were also detected in both isolates. Finally, transformation studies using donor genomic DNA from these 2 isolates were performed on *H. influenzae* Rd KW20. While none of the transformants that were generated exhibited as high an azithromycin MIC as the donors, a number of different regions of donor origin were detected in various transformants. The role of these regions in generating resistance in individual transformants was not clear but, with respect to the lower MICs exhibited by these transformants, the findings suggested a multifactorial aetiology for the high-level macrolide resistance seen in the donor isolates.

In the Roberts et al. study, the effect of the AMRGs appeared to be inconsistent, with some isolates not exhibiting increased MICs (compared to a typical wild-type strain). The effect of these AMRGs on macrolide susceptibility in *H. influenzae* remains to be established. As a result, the aim of Chapter 6 was to transfer select AMRGs (*erm*(A), *erm*(B), *erm*(C), *mef*(A) and *mef*(E)) to *H. influenzae* Rd KW20 and examine the phenotypic effect of expression of these genes. Initially, attempts were made to conjugatively transfer these AMRGs from select Gram positive donors to *H. influenzae* Rd KW20. These attempts were unsuccessful. As a result, the AMRGs were cloned into the shuttle vector pLS88 and *H. influenzae* Rd KW20 was transformed with these constructed plasmids by electroporation. Clones were generated with a range of approaches, including with and without the native regulatory regions of the AMRG inserts, and in the former, tested for expression with and without the presence of an inducing agent. High-level expression of *erm*(A), *erm*(B) and *erm*(C) was demonstrated in at least some of the various conditions and resulted in increased macrolide resistance in these transformants. In contrast, expression of *mef*(A) and *mef*(E) did not have an effect on macrolide resistance. In the Roberts et al. study, conjugative transfer of *mef*(A) to *H. influenzae* Rd KW20 resulted in a moderate increase in azithromycin and erythromycin MICs; our findings therefore suggest that *mef* only increases MICs in combination with other macrolide resistance mechanisms such as *msr*(D) (found downstream of *mef* and not covered by our cloned inserts) or underlying chromosomal alterations.

The above AMRGs are commonly encountered among human pathogens which share a respiratory niche with NTHi, including *Staphylococcus aureus* and various respiratory *Streptococcus* spp.. A number of other AMRGs, such as *erm*(42), *msr*(E) and *mph*(E), are typically encountered among animal commensals and pathogens such as *Pasteurella* spp. and *Mannheimia* spp.. *H. influenzae* is closely related to these pathogens and previous studies have demonstrated that they are able to exchange resistance determinants, including beta-

lactamases. Therefore, the work of Chapter 7 explored the potential for conjugative transfer of *erm*(42), *msr*(E) and *mph*(E) (carried on the mobile multiresistance integrative and conjugative element ICE*Pmu1*) and associated macrolide resistance from a bovine *Pasteurella multocida* isolate to *H. influenzae* Rd KW20. Transconjugants generated in this study were found to carry a truncated form of ICE*Pmu1* that lacked *msr*(E) and *mph*(E) but carried *erm*(42); transconjugants were found to exhibit increased erythromycin and clindamycin resistance. This truncated ICE*Pmu1* was successfully transferred from primary transconjugants to secondary *H. influenzae* Rd KW20 recipients, indicating that transfer functions were retained during conjugation. The acquisition of ICE*Pmu1* did not appear to have an impact on the fitness of *H. influenzae* Rd KW20 and the ICE was found to be stable in the absence of antibiotic selective pressure.

In summary, the major findings of this thesis are that AMRGs are not widespread in NTHi and that the high prevalence of a selected set of these genes described in one recent study is probably unique to the circumstances of that study. Although complex regulatory regions in many AMRGs mean that expression and associated resistance may be dependent on the specific genetic context, we have shown that under favourable conditions, *erm*(A), *erm*(B) and *erm*(C) can produce macrolide resistance in *H. influenzae* in their own right, but this is unlikely for the *mef* genes. Although we were unable to demonstrate conjugative transfer of common AMRGs from respiratory Gram positive organisms to *H. influenzae*, we were able to demonstrate conjugative transfer of an AMRG encoding multi-resistance replicon from a closely related organism of animal origin. This replicon produced macrolide resistance, was stably maintained without a significant fitness cost and was capable of ongoing conjugative transfer within *H. influenzae*. This thesis concludes that there is little evidence for the imminent and widespread emergence of AMRG associated macrolide resistance in NTHi; however, the increasing use of macrolides in both animals and humans and the prevalence of

AMRGs in other organisms suggest that it is prudent to undertake ongoing periodic surveillance for these genes in NTHi.

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Chapter 1: Summary of Thesis

1.1 Background

Respiratory tract infections (RTIs), including those associated with chronic obstructive pulmonary disease (COPD), are among the most common infections encountered in Australia and globally. The bacterial pathogen nontypeable *Haemophilus influenzae* (NTHi) is frequently implicated as a cause of these infections. Antibiotic therapy has historically been utilised to treat RTIs associated with NTHi, with the β-lactam antibiotics amoxicillin, amoxicillin-clavulanate and cefuroxime (or other appropriate oral cephalosporins) commonly prescribed to these patients. Macrolide antibiotics such as erythromycin, clarithromycin and azithromycin are being increasingly prescribed for COPD-related RTIs, and while many strains of NTHi exhibit reduced susceptibility to macrolides compared to Gram positive organisms due to the presence of an inherent intrinsic efflux system, macrolides are commonly prescribed for infections where NTHi may be involved.

High-level macrolide resistance mechanisms in bacteria can be categorised into two broad groups. Firstly, resistance can occur due to mutations in particular structural elements of the bacterial ribosome, including within 23S rRNA, and the L4 and L22 ribosomal proteins. Secondly, resistance has been attributable to the acquisition of macrolide resistance genes (AMRGs) which code for a variety of resistance mechanisms, including enzymes which alter the ribosomal binding site of the macrolide (such as those coded by the *erm* genes), and alternate efflux systems (including those encoded by the *mef* genes).

AMRGs have been widely documented among Gram positive bacteria in particular, including among respiratory organisms that frequently co-exist with NTHi in the respiratory tract, such as *Streptococcus pneumoniae*. However, high-level macrolide resistance in NTHi has

historically been attributed to the presence of mutations within L4, L22 and 23S rRNA. By contrast, the role of AMRGs in causing macrolide resistance in NTHi remains controversial; while a number of previous studies have failed to detect these genes in *H. influenzae* isolates exhibiting high-level macrolide resistance, a more recent study by Roberts et al. (2011) reported the presence of these genes in large numbers among a collection of NTHi isolates of variable macrolide resistance phenotype derived from children with cystic fibrosis enrolled in a placebo-controlled azithromycin trial. As a result, the research presented in this thesis aims to address the scientific gaps in the understanding of the prevalence, role and potential acquisition of AMRGs among respiratory isolates of NTHi.

1.2 Chronology of Works and Thesis Organisation

This chapter of the thesis (**Chapter 1**) comprises a general introduction into the overall themes and rationale of the thesis along with a note on the chronology of the laboratory works conducted.

Chapter 2 presents a review of the current literature that summarises the background information on the main themes of the thesis. This includes a general overview of macrolide structure, function and use, an overview of *H. influenzae* as a bacterial pathogen (bacteriology and pathogenesis), and the prevalence of macrolide resistance among key respiratory organisms. Subsequently, Chapter 2 provides a detailed review of the macrolide resistance mechanisms that have been documented, including the various AMRGs currently described in the literature as well as chromosomal mutations within ribosomal structural elements. This section is first considered broadly among the global bacterial population, and is then narrowed down to focus on NTHi and other phylogenetically related organisms. Finally, this chapter finishes with the specific aims of the thesis.

Chapter 3 describes general methodologies that were applied throughout multiple studies of this thesis, including culture media, extraction of genetic materials from study strains (DNA and RNA), and genetic sequencing methodologies.

Chapter 4 presents the first study of this work, which aimed to investigate the potential presence of select AMRGs in clinical respiratory isolates of NTHi through the replication of the study performed by Roberts et al. (2011).

Particular components of this work were published as:

Atkinson CT, Kunde DA, Tristram SG (2015). Acquired macrolide resistance genes in *Haemophilus influenzae*?. *J Antimicrob Chemother*; 70 (8): 2234-6. [doi: 10.1093/jac/dkv093].

The findings from this study were that the AMRGs chosen for this work were not present among the established collection of NTHi isolates, which correlates with previous studies demonstrating that these genes are not widespread among *H. influenzae*, and that the findings reported by Roberts et al. (2011) may have been unique to the circumstances of that particular study.

Chapter 5 presents the second study of this work, which aimed to utilise whole genome sequencing techniques to further investigate the presence of a larger assortment of AMRGs among a collection of publically available whole genome sequences of NTHi and to detect other potential macrolide resistance mechanisms in isolates of NTHi exhibiting high-level macrolide resistance from our collection. The findings of this study further supported previous studies and the findings of Chapter 4 of this thesis (through the inability to detect AMRGs among the collection of whole genome sequences included in this study) that the acquisition of AMRGs is not a widespread phenomenon in NTHi. The high-level macrolide

resistance detected in the relevant isolates included in this study was attributed to chromosomal mutations in 23S rRNA and L22; a number of other potential mechanisms were identified through the utilisation of transformation assays performed for this chapter, although the role of these has not been established.

Chapter 6 presents the third study of this thesis, which aimed to investigate the phenotypic effect of AMRGs in *H. influenzae* through the utilisation of conjugation and cloning assays to generate recombinant strains with AMRGs, and expression assays and antibiotic resistance testing to examine the effect of the AMRGs. The novel findings from this study were that expression of various *erm* genes in *H. influenzae* resulted in a clear reduction in susceptibility to macrolides, with *erm*(B) and *erm*(C) having a greater effect than *erm*(A). By contrast, expression of *mef* did not appear to have an appreciable effect on susceptibility, which did not match the findings of Roberts et al. (2011), although further work is required to clarify this finding due to the potential co-operative role of *msr*(D).

Chapter 7 presents the final study of this thesis, which aimed to investigate an alternate potential pathway of AMRG acquisition by *H. influenzae* by way of conjugative transfer from animal-derived but phylogenetically related organisms such as *Pasteurella multocida*. The novel findings of this study demonstrated successful transfer of *erm*(42) (but not of *msr*(E) and *mph*(E)) to *H. influenzae* Rd KW20 on the integrative and conjugative element (ICE) ICE*Pmu1*, with an associated decrease in macrolide susceptibilities. ICE*Pmu1* was shown to be a stable element which had minimal impact on the fitness of *H. influenzae* Rd KW20 and retained its transfer functions in the transconjugants.

Chapter 8 presents a general discussion and concluding remarks of the major findings from the studies of this thesis. Potential limitations of the study design that were not previously

addressed in the prior chapters and justifications of the designing of the studies are also discussed here.

A complete reference list is presented in **Chapter 9** of this thesis. The referencing style used for this thesis conforms to the style of the Journal of Antimicrobial Chemotherapy. Finally, two appendices are presented at the end of the thesis. **Appendix 1** contains PDF reproductions of published letters by Roberts et al. (2015) and Atkinson et al. (2015) that were produced in response to the original manuscript published based on the work presented in Chapter 4 of this thesis (see Chapter 4 section above for reference details for the original manuscript). **Appendix 2** contains supplementary data for the work described in Chapter 5 of this thesis.

Chapter 2: General Introduction and Review of the Literature

2.1 Macrolide structure, binding and function

2.1.1 Macrolide structure

The macrolide antibiotics are compounds that are characterised by the presence of a macrocyclic lactone ring and the size of the lactone ring varies between the different macrolides. Erythromycin, roxithromycin, and clarithromycin have a 14-membered lactone ring, azithromycin has a 15-membered ring, and josamycin, carbomycin, and tylosin have a 16-membered ring.[1,2] To this lactone ring are attached a number of deoxy sugars, via glycosidic bonds, which differ for each macrolide, although in general a mono- or poly-saccharide side chain will occur at atom C5 of the lactone ring (note that numbering starts at the macrolide ester bond, see Figure 2.1).[1] The different sizes of the lactone ring and the different sugar moieties attached to this ring give each macrolide distinct properties and efficacies. As an example of the different structures of various macrolides, erythromycin and clarithromycin both have a cladinose attached at position C3 and desosamine at position C5, but are distinguished by the substitution of the C6 hydroxyl group (erythromycin) for a methoxy group (clarithromycin). Azithromycin also has these sugars attached, but is made into a 15-membered macrolide by the addition of a nitrogen atom into the lactone ring. Roxithromycin shares structural similarities with erythromycin, but the oxygen bound at C9 in erythromycin is replaced with an N-oxime side chain.

2.1.2 The bacterial ribosome, macrolide binding and antibiotic properties

The ribosome is an organelle involved in the translation of mRNA into proteins.[3] The ribosome is made up of rRNA and dozens of distinct proteins that are arranged into smaller subunits and active sites. The 2 major subunits of the bacterial ribosome are the smaller 30S

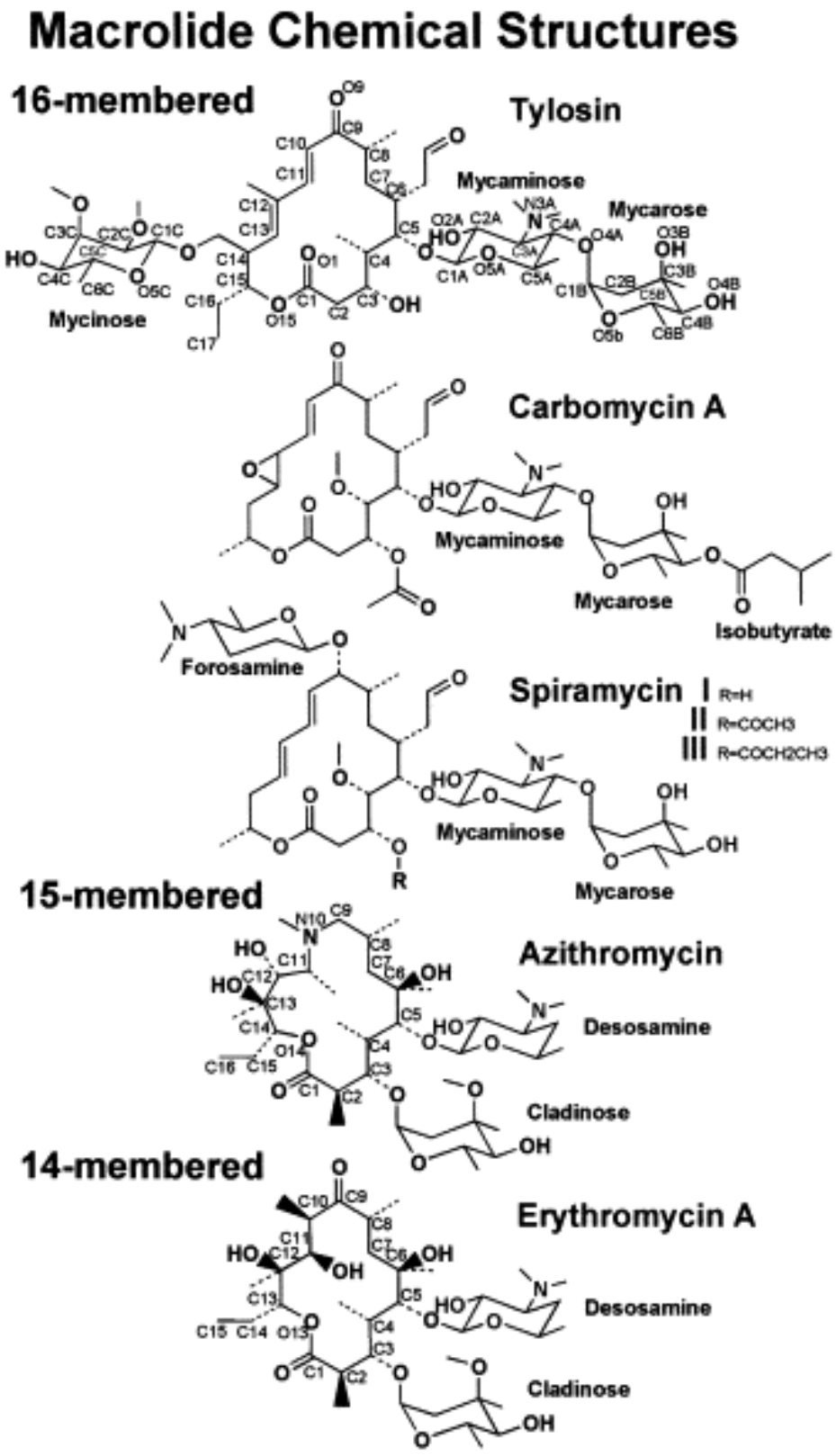

Figure 2.1: Example of the differing chemical structure of particular macrolides. Each macrolide has a lactone ring to which sugar substituents are attached.[4]

subunit and the larger 50S subunit. The 16S subunit exists within the 30S subunit, and the 5S and 23S subunits exist within the 50S subunit. Three active sites exist in the ribosome: the P-site (in which peptidyl tRNA is formed and in which the first aminoacyl tRNA enters), the A-site (the point of entry of subsequent aminoacyl tRNA), and the E-site (the exit site of tRNA after it has delivered its amino acid to the polypeptide chain).

Protein synthesis is initiated by the binding of mRNA to the 30S subunit. During the process of polypeptide elongation, the ribosome undergoes several conformational changes that allow for the entry and accurate recognition of the appropriate type of tRNA. Elongation begins with the entry of the first aminoacyl tRNA (carrying fMET) into the P-site, which initiates a conformational change allowing for more aminoacyl tRNA (carrying the next amino acids in the protein sequence) to enter the A-site. At this point, peptide bond formation between the last amino acids in the polypeptide (which is detached from the P-site tRNA) and the following amino acid (still attached to tRNA in the A-site) occurs. After this, the uncharged tRNA moves to the E-site to be released from the ribosome, the polypeptide in the A-site moves into the P-site, and a new codon moves into the A-site. This process is known as translocation. Elongation continues until a stop codon is recognised, with the growing protein exiting the ribosome through the larger subunit of the ribosome.

Macrolides are protein synthesis inhibitors that have long been valued for their antibiotic properties ever since erythromycin was discovered in the 1950s.[1] Macrolides have broader activities than penicillins and are less likely to produce an allergic response.[5] In general, macrolides bind to the larger 50S subunit of the ribosome, inside the peptide exit tunnel, making contact with hairpin 35 in domain II and the peptidyl transferase loop in domain V (these two regions act as the binding pocket).[6] This can result in a number of effects, including the inhibition of peptide bond formation,[7] interference with 50S assembly,[8] and early dissociation of peptidyl tRNA from the ribosome during translocation.[9]

Macrolides and their derivatives interact with the bacterial ribosome in a number of ways (see Figure 2.2) and the way in which these drugs interact with the ribosome depends on the sugars attached to the lactone ring and can also affect the mechanism in which they inhibit protein synthesis.[10] For example, erythromycin does not affect peptide bond formation, but has been shown to affect 50S assembly[8] and can promote early dissociation of peptidyl tRNA.[9] The mono- or polysaccharide side chain of C5 forms an important contact with rRNA. In the case of erythromycin and its numerous derivatives, the desosamine sugar forms hydrogen bonds with nitrogen bases located at the nucleotide residues A2058 and A2059 (note that the nucleotide numbering used here is based on that of *Escherichia coli*).[10] The base pair 2611-2057 (particularly the latter nucleotide of this pair) and the backbone phosphate oxygen of G2505 may also be involved with macrolide binding.[10] In the case of the larger 16-membered macrolides such as carbomycin and spiramycin, the mycaminose-mycarose forms a hydrogen bond with A2058 along with a number of mostly hydrophobic interactions.[10] In addition, the acetaldehyde group at C6 of these 16-membered macrolides appears to form a reversible covalent bond with A2062.[10] Ketolides, derived from erythromycin by the substitution of cladinose for a 3-keto,[6] can also interact with the loop of helix 35 in domain II of 23S rRNA if a ketolide extension (an alkyl-aryl chain from a carbamate group) is present at position 11 or 12 of the lactone ring, specifically with nucleotide A752.[6]

In addition, the L4 and L22 ribosomal proteins are located within the peptide exit tunnel of the 50S subunit, which is otherwise lined with RNA loops.[3] Macrolides bind in a narrow part of this tunnel between the peptidyl transferase centre and a constriction in the tunnel near L4 and L22.[4] It is known that some macrolides are able to interact with these ribosomal proteins. For example, the forosamine moiety of spiramycin reaches L4, while the mycinose sugar of tylosin is able to interact with L22.[4] Azithromycin and other smaller macrolides do not

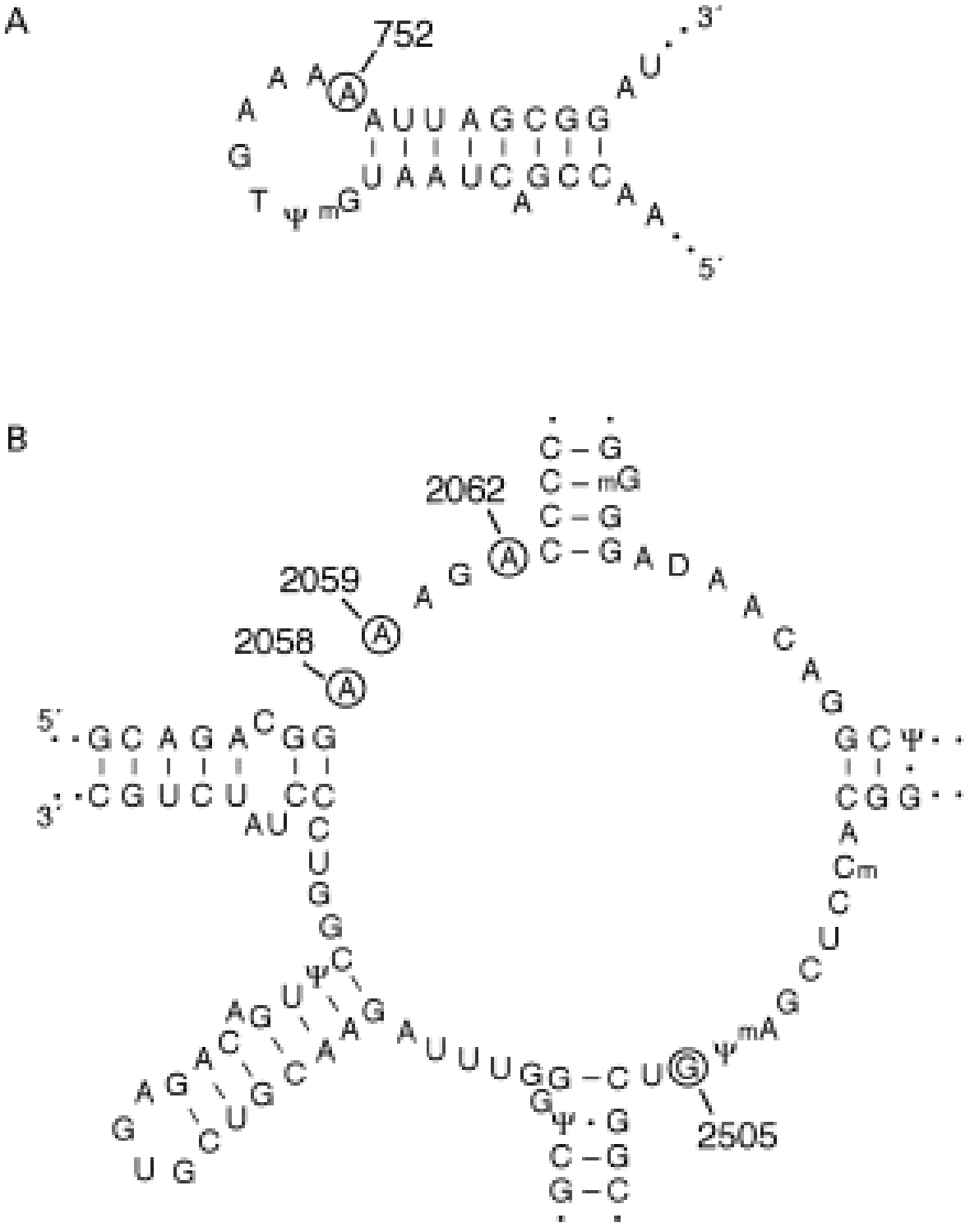

Figure 2.2: Hairpin 35 (A) and the peptidyl transferase centre (B) of 23S rRNA. Circled nucleotides represent particular macrolide (including derivative drugs) interaction sites.[6]

appear to make contact with these proteins however.[4]

The macrolide drug class displays a degree of overlap with other drug classes based on where it binds on the ribosome (the peptidyl transferase centre of the 50S subunit) and the resulting effects of this binding. This overlap occurs with the lincosamide antibiotics and streptogramin B.[1] Despite being chemically distinct from one another, the overlap means that macrolides often have similar activities to lincosamides and streptogramin B, and they are therefore considered together as a superfamily. This group has traditionally been referred to using the umbrella term MLS_B.[11] In some instances, the MLS_B group has also been grouped with the ketolides (larger derivatives of macrolides) and the oxazolidinones for similar reasons; this group is referred to as MLSKO.[1]

Erythromycin has traditionally been more effective against Gram-positive organisms, particularly staphylococci and streptococci. Gram-negative organisms are typically more resistant to erythromycin. Derivatives of erythromycin such as azithromycin, clarithromycin and roxithromycin have an expanded spectrum of activity that provides better coverage against particular groups of organisms and exhibit superior stability.[12] Erythromycin and azithromycin tend to have a similar spectrum of activity against Gram-positive organisms, but azithromycin has stronger activity against Gram-negative organisms. Clarithromycin is less active than azithromycin against Gram-negative organisms (although still superior to erythromycin in this respect), but tends to have a broader spectrum of activity against Gram-positive organisms. Roxithromycin is more stable in the presence of gastric acid than erythromycin and has improved activity against particular organisms including *Pasteurella multocida* and *H. influenzae*.

2.1.3 Macrolides as immunomodulatory and anti-inflammatory agents

Distinct from their direct antibacterial effects, it has also been demonstrated that macrolides have the ability to modulate the immune response[13] at sub-inhibitory doses.[14] In general, 14- and 15-membered macrolides have similar immune-modulatory effects, while 16-membered macrolides lack these effects.[14] The modulation effects of macrolides are broad. Macrolides are known to be able to exert inhibitory effects on immune signalling components. For example, erythromycin, clarithromycin, and roxithromycin all inhibit NF-κB, a protein complex involved in the upregulation of genes involved in T-cell production and function (see Figure 2.3).[14-16] Macrolides have also demonstrated inhibitory effects on acute phase response proteins released during inflammation, including IL-1, IL-8, and TNF-α.[14]

Furthermore, evidence suggests that some macrolides have the ability to upregulate the production of β-defensin-1 and β-defensin-2 on the surface of human airway epithelial cells, reducing the susceptibility of the airways to bacterial infection.[17,18] Macrolides are also able to stimulate phagocytosis and enhance mannose receptor expression on macrophages, facilitating the elimination of bacterial pathogens.[17,19]

Macrolides have been shown to exert effects on a variety of cell lines; notably, macrolides are able to upregulate apoptosis in neutrophils, eosinophils, and lymphocytes.[14] Furthermore, macrolides can limit the tissue damage of neutrophil action through the inhibition of granulation and the respiratory burst.[14] This is believed to be beneficial in the management of chronic pulmonary inflammatory diseases, although the benefits of macrolides in managing acute inflammation are less clear.[20]

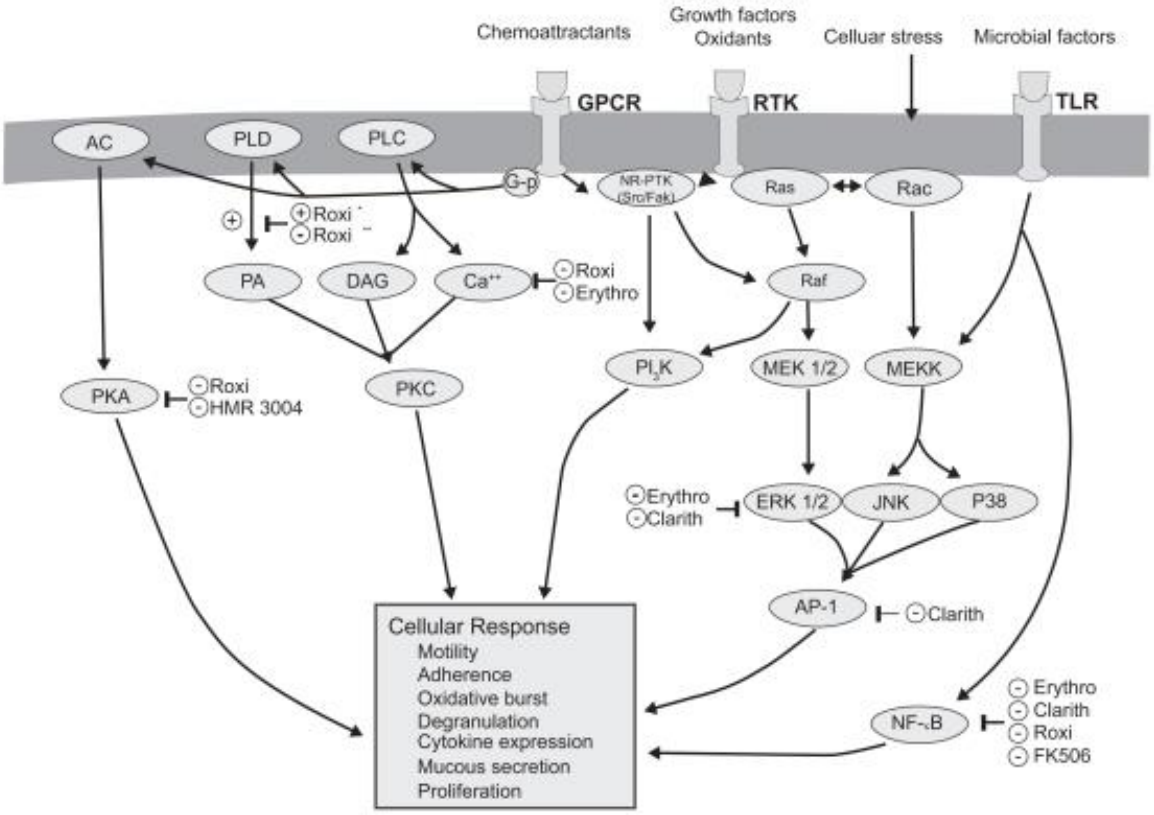

Figure 2.3: Inflammatory molecular targets of macrolides. Macrolides can exert their effects on several targets, including NF—κB.[21]

2.2 Clinical applications of macrolides in respiratory infections

Macrolide have been used for decades in the clinical setting, and they remain among the most commonly prescribed drug classes because of their antibiotic and anti-inflammatory properties. Macrolides are commonly prescribed for respiratory infections, particularly those where inflammation is a likely contributing factor. The following section describes some of these conditions.

2.2.1 Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a condition defined by persistent airflow deficiency as a result of emphysema (lung tissue breakdown), and is characterised by a chronic cough, excessive sputum production, and shortness of breath. COPD affects 5% - 10% of adults globally,[17] and was recognised as the sixth leading cause of death in the world in 2008.[14] Up to one in five Australian's over 40 are believed to be affected by COPD;[22] in 2008, COPD was estimated to cost the Australian economy an estimated $98 billion.[23] Tobacco smoke is recognised as the most common trigger for the COPD-associated inflammatory response in the lung. Patients with a diagnosis of COPD often experience exacerbations of their symptoms, including increasing shortness of breath, and changes in sputum production and consistency. These exacerbations are frequently attributable to bacterial and viral infections; *H. influenzae*, *S. pneumoniae*, *Moraxella catarrhalis*, and *S. aureus* are among the more common bacterial causes.

The disease process is known to involve neutrophil and eosinophil activation, and the inflammatory markers involved in this activation tend to be elevated in the sputum of patients during an exacerbation.[5] Management of COPD involves reducing the frequency of these exacerbations.[17] Although the ability of macrolides to control airway inflammation has been known for some time,[5] there is limited data available on the effectiveness of long-term

administration of macrolides in the management of COPD. Both erythromycin and azithromycin have been proposed as reducers of the frequency of exacerbations in many patients,[24,25] but the exact mechanism that causes this is unclear.[26] One theory suggests that the stimulatory effect of macrolides on phagocytosis may be an important factor. Notably, defective alveolar macrophages and impaired monocyte-derived macrophage phagocytosis are common features of COPD,[27,28] so macrolide administration may counter these effects.[17]

While macrolides may be an effective management tool of the inflammatory aspects of COPD, more recent evidence suggests that macrolides may not be as effective in eradicating particular bacterial species associated with acute exacerbations in COPD patients. Pettigrew et al. (2016) reported that macrolide therapy did not appear to be effective in eradicating *H. influenzae* in COPD patients, and that it may in fact encourage the emergence of macrolide-resistant *H. influenzae* isolates in these patients. By contrast, fluoroquinolones appeared to be more effective in eradicating *H. influenzae* in these patients.[29]

2.2.2 Cystic fibrosis

Cystic fibrosis (CF) is the most common fatal genetically inherited disease in Caucasians, with highest prevalence in Europe, North America and Australia.[30] The disease occurs in approximately 1 in 3000 live births.[31] The disease is caused by defects in the *CFTR* gene, which results in impaired salt and water transport across epithelial surfaces.[30,32] Although this affects several organs, the most apparent effects are within the respiratory system. The gene mutation ultimately leads to unusually thick secretions, and patients are unable to clear their lungs of bacteria-containing mucus as a result. Therefore, CF patients develop chronic pulmonary infections and associated inflammation, ultimately resulting in respiratory failure and death in many cases.[33]

The bacterial species that cause these infections appears to be age-dependent (see Figure 2.4). In younger patients, the predominant organisms are *S. aureus*[34] and *H. influenzae*.[32] As patients grow older, these organisms are less commonly isolated in favour of *Pseudomonas aeruginosa*,[34] although there is contention as to whether this is because of true replacement by *P. aeruginosa* or simply because standard laboratory techniques are unable to detect these "childhood" organisms in the presence of the far more easily isolated *P. aeruginosa*.[35]

There has been recent interest in using macrolide antibiotics to treat CF patients, which has direct killing action against many of the common pathogens in childhood CF-associated infections.[34] Although *H. influenzae* is relatively insensitive to macrolides and *P. aeruginosa* is typically resistant to azithromycin, there is some speculation that the drug may still benefit adult patients through its anti-inflammatory actions. Furthermore, it has been proposed that azithromycin may be able to inhibit the production of certain virulence factors associated with *P. aeruginosa* infection, such as bio-film formation.[34]

2.2.3 Pneumonia

Community-acquired pneumonia (CAP) is a leading cause of morbidity and mortality globally; treating the disease carries a significant cost burden on the world's health systems. CAP can affect individuals of all ages, with infants and the elderly being particularly susceptible.[36] Among the more common bacterial causes of CAP are *S. pneumoniae*,[37] and NTHi.[38] Because of their broad coverage, azithromycin and clarithromycin are among the more highly recommended antibiotics for treating CAP in adults where no other treatments have been previously administered.[37] In cases of hospital-acquired pneumonia, *S. aureus* and *P. aeruginosa* are more commonly isolated than species normally seen in CAP.[39] Macrolides are less frequently used in nosocomial pneumonia cases, but some clinicians may opt to add a macrolide to treat these patients for coverage against atypical pathogens and for their

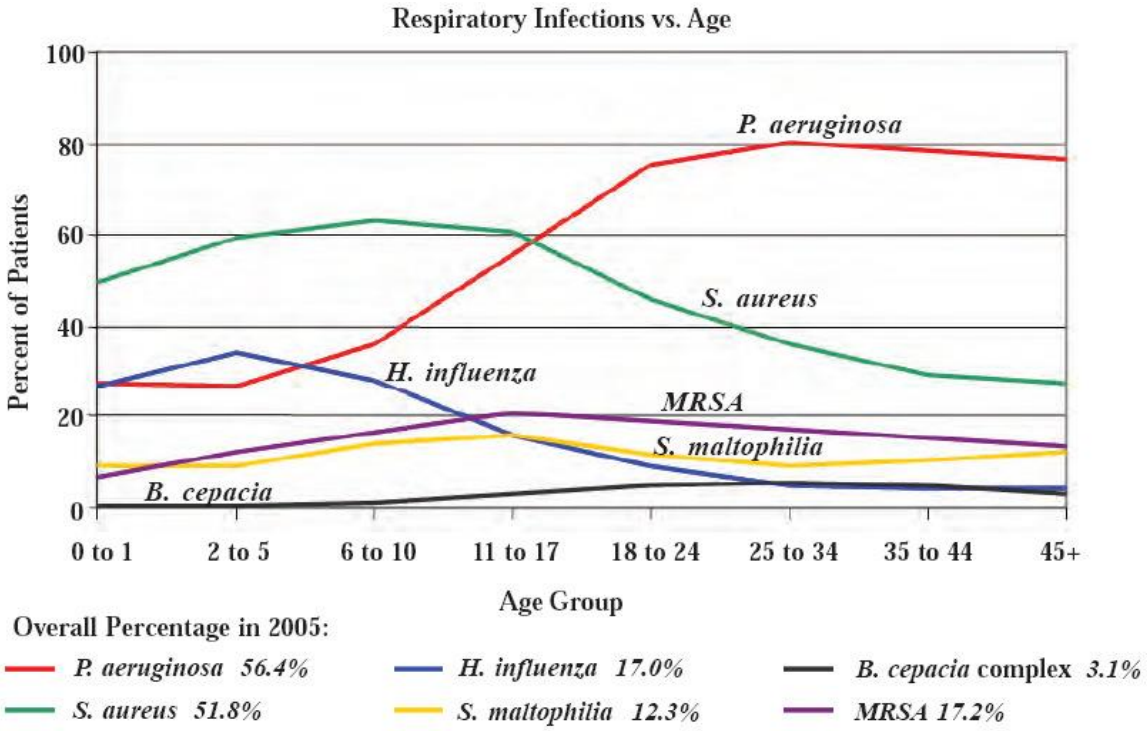

Figure 2.4: The relationship between CF patient age and the causative agent of respiratory infection.[40]

anti-inflammatory effects.[39]

2.2.4 Pharyngitis ("strep throat")

Pharyngitis is a common and generally non-life threatening condition characterised by inflammation of the pharynx. The majority of acute cases are viral in aetiology, although bacterial causes are also frequently encountered.[41] *Streptococcus pyogenes*, also referred to as group A streptococcus (GAS), has historically been identified as the most common cause of bacterial pharyngitis,[41] with such cases commonly referred to as "strep throat". Streptococcal pharyngitis is more commonly encountered in children, accounting for around 15-30% of cases of acute pharyngitis, although adults also commonly present the condition.[41] Less common and less widely recognised and accepted causes of bacterial pharyngitis include *S. pneumoniae* and *H. influenzae*.[42]

The condition is often self-limiting but antibiotic therapy is commonly used to manage bacterial pharyngitis. β-lactam antibiotics such as penicillin and amoxicillin are considered the antibiotic of choice for several reasons. Aside from *S. pyogenes* remaining susceptible to β-lactams overall, the drug class has a narrow spectrum of activity and is effective in preventing post-streptococcal sequelae, including rheumatic fever.[43] In cases where β-lactams are not an appropriate choice (such as where the patient has an allergy to β-lactams), azithromycin and clarithromycin have been recommended as first-line alternatives, although macrolide resistance prevalence has increased among *S. pyogenes*, and cephalosporins have been investigated as another alternative to β-lactams.[41,43,44]

2.2.5 Asthma and other inflammatory disorders

Asthma affects 1 in 10 adults in Australia, and up to 1 in 9 children.[45] Exacerbations in asthma patients, which are primarily neutrophilic, are often attributable to bacterial or viral infection, and patients with asthma are prone to developing persistent and highly

symptomatic inflammatory responses upon rhinovirus infection.[5] The first macrolide used to treat asthma was troleandomycin in the 1960s, but its adverse effects on the hepatic system were undesirable.[46] Telithromycin has been proposed as a safer option for relieving the effects of exacerbations in asthma patients,[46] while azithromycin and clarithromycin have been indicated as useful tools in the management of chronic asthma.[47,48]

Research is continuing into the application of macrolides in other inflammatory conditions. A 1998 study demonstrated improvement in the conditions of patients with diffuse panbronchiolitis after taking low-dose erythromycin,[49] while evidence is suggestive of the possible benefits for azithromycin, clarithromycin, erythromycin use in non-CF-associated bronchiectasis.[46] There is also evidence for the potential benefits of azithromycin use in patients who develop bronchiolitis obliterans following a lung-transplant.[46]

2.2.6 *Haemophilus influenzae* and macrolides

H. influenzae is a fastidious, pleomorphic, Gram-negative bacterial species that is said to be "human-specific", in the sense that humans form the only known reservoir.[50] *H. influenzae* is distinguished from most other *Haemophilus* spp. by its characteristic dependence on haemin (factor X) and nicotinamide adenine dinucleotide (NAD, or factor V) for growth.[51] It grows best at 37°C in a 5% CO_2 environment.[50]

H. influenzae can be separated into 2 subgroups based on the presence or absence of a polysaccharide capsule. The ability to produce a capsule is among the most important virulence factors in *H. influenzae* as it facilitates survival, most notably within the blood stream (thus allowing these strains to cause invasive disease).[51] The encapsulated strains produce mucoid colonies when cultured and are further distinguished into serotypes "a" to "f" based on the antigenic profile of the capsule, with serotype "b" (commonly referred to as Hib) being the most clinical significant serotype.[51] Hib is frequently involved in serious

infections in childhood and is a known cause of a variety of diseases, including meningitis[51,52] and pneumonia;[51,53] at one point, Hib accounted for 95% of all reported *H. influenzae* disease.[54] The incidence of Hib disease in the developed world has dropped dramatically since the introduction of the Hib vaccine in the 1970s[51,55] and other serotypes have become more commonly associated with opportunistic invasive disease.[56,57] For example, serotypes a, d and f are linked to cases of bacteraemia and pneumonia in immunocompromised adults. Hib remains a significant threat in countries where routine Hib vaccination has not been introduced, however.[56]

The non-encapsulated strains are referred to as NTHi. They are non-mucoid on culture and are frequently isolated as normal flora of the human respiratory tract, with colonisation typically occurring early in life.[58] While NTHi isolates are considered less pathogenic than the encapsulated variants, NTHi has been identified as an important cause of number of acute opportunistic respiratory infections in children and adults.[53] NTHi has been attributed to causing between 2 and 12% of cases of community-acquired pneumonia (CAP) in adults,[37] and is also an uncommon cause of hospital-acquired pneumonia.[59] Furthermore, NTHi is the second-most common cause of acute otitis media (AOM), behind *S. pneumoniae*,[60] causing between 23 and 67% of cases.[37] NTHi also plays a role in acute and chronic cases of sinusitis,[60] with 35% of acute episodes in adults and 28% in children being attributable to NTHi.[61] NTHi has been acknowledged as among the most common bacterial causes of acute exacerbations of chronic bronchitis (AECB) in patients with COPD.[37] Note that while NTHi is considered less invasive than the encapsulated strains, the reduced incidence of Hib has resulted in NTHi now being recognised as an opportunistic invasive pathogen that has been implicated in meningitis and bacteraemia in immunocompromised children and adults with underlying medical conditions.[57,60]

Compared to other respiratory pathogens, NTHi tends to be less susceptible (although not necessarily completely resistant) to the antimicrobial effects of macrolides.[37] This lower baseline susceptibility is believed to be due to an inherent efflux mechanism that has activity against macrolides (see section 1.5 for more information on this topic). In any case, macrolides such as azithromycin and roxithromycin continue to be used in situations where NTHi may be involved, including the conditions described previously in this review. Given their immunomodulatory and anti-inflammatory properties, macrolides may still be clinically beneficial even in situations where NTHi may itself not be responsive to the drug.

2.3 Macrolide resistance prevalence among key respiratory bacteria

The focus of this thesis will be to investigate the role that acquired macrolide resistance genes (AMRGs) currently play, or may play in the future, in macrolide resistance in NTHi. In order to assess the potential for the transfer of AMRGs to NTHi to occur in the upper respiratory tract, it is necessary to examine the prevalence of macrolide resistance in common respiratory isolates known to frequently harbour AMRGs. For the purposes of this review, the prevalence of macrolide resistance in *S. pneumoniae*, *S. pyogenes* and *S. aureus* will be examined because the macrolide resistance in these organisms is commonly associated with AMRGs, and these organisms are both frequent upper respiratory commensals that will regularly co-exist with NTHi, and pathogens for which a large body of susceptibility data exists.

Within just years of the introduction of erythromycin as an antibiotic in 1952, erythromycin-resistant bacterial isolates had emerged.[62] The increasing use of erythromycin and in particular its derivatives like azithromycin and clarithromycin for managing infection and inflammation has led to an increase in the proportion of macrolide-resistant isolates among both Gram-positive and Gram-negative species;[63] by the 1990s, resistance had become widely disseminated and had been detected in isolates of *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Mycoplasma pneumoniae*, *Campylobacter* spp., *Propionibacterium* spp., and various members of the family *Enterobacteriaceae*.[62] The risks associated with increasing macrolide resistance rates are substantial, but it should be said that the risks are not necessarily great to the individual patients taking them.[26] Rather, there is an emerging issue regarding the effects of long-term macrolide use to the community at large. Because infecting bacteria rarely exist alone in the lungs, but are also in the presence of "innocent

bystander" organisms, any macrolide therapy used to treat the target organism can result in macrolide exposure to these "bystanders" as well. These organisms are generally transmitted to other people in the community rather easily, and they may potentially transfer any acquired macrolide resistance mechanisms to other organisms. This means that if long-term macrolide therapy is undertaken by a significant proportion of the community, the proportion of resistant organisms circulating within the community may also increase.[26,64]

A large number of studies have been conducted that have analysed the overall prevalence of macrolide resistance among respiratory pathogens. Much of the available data is based on phenotypic resistance, although it must be stressed that resistance in any one isolate based on minimum inhibitory concentration will not necessarily by clinically useful. Difficulties are presented when attempting to define "resistance" in this case because there is no consensus as to which macrolide/s should be used to determine whether an isolate is resistant to the drug class as a whole. Furthermore, there is often a lack of consensus for the resistance breakpoints used to define resistance in particular species provided by the more commonly referenced antibiotic susceptibility testing methods (see Tables 2.1-2.4). These differences can be attributed to the variable processes in which these breakpoints are determined.[65] Regardless of these issues, the data obtained from surveillance studies such as the Alexander Project and the PROTEKT surveillance study made it clear that the prevalence of macrolide resistance in particular species was increasing in response to the increasing use of macrolides for treating infections.

2.3.1 *Streptococcus pneumoniae*

In *S. pneumoniae*, where macrolide resistance rates have been well documented, macrolide resistance prevalence increased at an alarming rate throughout the 1990s, and has been associated with the increased reliance on macrolides therapy.[66] In the United States, where

Table 2.1: Susceptibility breakpoints provided by commonly used susceptibility interpretation methods for *Streptococcus pneumoniae.*

Method	Erythromycin resistance breakpoints (MIC, µg/mL)			Azithromycin resistance breakpoints (MIC, µg/mL)			Clarithromycin resistance breakpoints (MIC, µg/mL)		
	S	I	R	S	I	R	S	I	R
Clinical and Laboratory Standards Institute (CLSI)[67,68]	≤0.25	0.5	≥1	≤0.5	1	≥2	≤0.25	0.5	≥1
European Committee on Antimicrobial Susceptibility Testing (EUCAST)[69]	≤0.25	0.5	>0.5	≤0.25	0.5	>0.5	≤0.25	0.5-1	>1
PK/PD[37]	≤0.25	----	≥0.5	≤0.12	----	≥0.25	<0.25	----	≥0.5

Table 2.2: Susceptibility breakpoints provided by commonly used susceptibility interpretation methods for *Staphylococcus aureus.*

Method	Erythromycin resistance breakpoints (MIC, µg/mL)			Azithromycin resistance breakpoints (MIC, µg/mL)			Clarithromycin resistance breakpoints (MIC, µg/mL)		
	S	I	R	S	I	R	S	I	R
Clinical and Laboratory Standards Institute (CLSI)[67,68]	≤0.5	1-4	≥8	≤2	4	≥8	≤2	4	≥8
European Committee on Antimicrobial Susceptibility Testing (EUCAST)[69]	≤1	2	>2	≤1	2	>2	≤1	2	>2
PK/PD[37]	≤0.25	----	≥0.5	≤0.12	----	≥0.25	<0.25	----	≥0.5

Table 2.3: Susceptibility breakpoints provided by commonly used susceptibility interpretation methods for *Streptococcus pyogenes.*

Method	Erythromycin resistance breakpoints (MIC, µg/mL)			Azithromycin resistance breakpoints (MIC, µg/mL)			Clarithromycin resistance breakpoints (MIC, µg/mL)		
	S	I	R	S	I	R	S	I	R
Clinical and Laboratory Standards Institute (CLSI)[67,68]	≤0.25	0.5	≥1	≤0.5	1	≥2	≤0.25	0.5	≥1
European Committee on Antimicrobial Susceptibility Testing (EUCAST)[69]	≤0.25	0.5	>0.5	≤0.25	0.5	>0.5	≤0.25	0.5	>0.5
PK/PD[37]	≤0.25	----	≥0.5	≤0.12	----	≥0.25	<0.25	----	≥0.5

Table 2.4: Susceptibility breakpoints provided by commonly used susceptibility interpretation methods for *Haemophilus influenzae.*

Method	Erythromycin resistance breakpoints (MIC, µg/mL)			Azithromycin resistance breakpoints (MIC, µg/mL)			Clarithromycin resistance breakpoints (MIC, µg/mL)		
	S	I	R	S	I	R	S	I	R
Clinical and Laboratory Standards Institute (CLSI)[67,68]	----	----	----	≤4	----	---	≤8	16	≥32
European Committee on Antimicrobial Susceptibility Testing (EUCAST)[69]	≤0.5	1-16	>16	≤0.125	0.25-4	>4	≤1	2-32	>32
PK/PD[37]	≤0.25	----	≥0.5	≤0.12	----	≥0.25	<0.25	----	≥0.5

macrolide use increased significantly from 1993 to 1999,[70] erythromycin resistance prevalence had increased from 0.2% (using CLSI breakpoints) in 1987 to 23% by 1999 (using CLSI breakpoints).[71,72] This increase in macrolide resistance prevalence in the United States was also observed in other studies. Doern et al. (2001), in a study that formed part of a longitudinal surveillance program, found erythromycin, azithromycin and clarithromycin resistance rates among a collection of 1531 clinical *S. pneumoniae* isolates exceeded 25% (using CLSI breakpoints) for all three drugs, representing an approximate increase of 16% over a period of 5 years ending in the winter of 1999-2000.[73] An overall rate of 27.9% (using CLSI breakpoints) was observed by a study by Farrell and Jenkins (2004) on a collection of 10012 *S. pneumoniae* isolates submitted as part of the PROTEKT study, with rates varying by state.[74] Pfaller et al. (2012), reporting on antibiotic resistance trends in the USA as part of the AWARE Ceftaroline Surveillance Program, reported an erythromycin non-susceptibility prevalence rate of 41.7% including both respiratory and bloodstream infection isolates of *S. pneumoniae* in 2010, a slight increase from the 38.4% prevalence rate recorded in 2008 (using CLSI methodology).[75]

This trend of increased resistance has also been observed globally. The overall prevalence of resistance to erythromycin, azithromycin and clarithromycin among a collection 8882 *S. pneumoniae* isolates submitted as part of the Alexander Project was shown to be approximately 25% (using CLSI breakpoints) by the turn of the century.[67] This study also showed that macrolide resistance was particularly prevalent in Asia, with overall rates approaching 70% for all 3 antibiotics. Resistance rates exceeded 80% (using CLSI breakpoints) in Hong Kong and 70% (using CLSI breakpoints) in Japan.[67] Resistance was also prevalent in parts of Europe, particularly in France (exceeding 50% for all 3 antibiotics using CLSI breakpoints), Italy (approximately 35% for all 3 antibiotics using CLSI breakpoints) and Spain (approaching 30% for all 3 antibiotics using CLSI breakpoints).[67]

Similar findings have also been observed in studies performed on isolates gathered from the PROTEKT surveillance study. Felmingham et al. (2002) found that among 3362 *S. pneumoniae* isolates, 31% were resistance to erythromycin. Again, resistance rates varied by country, with particularly high rates observed in Hong Kong (71.4%), Japan (77.9%), South Korea (87.6%), France (57.6%), Hungary (55.6%) and Italy (42.9%) (all using CLSI breakpoints).[76] In Australia, 12.3% (using CLSI breakpoints) of isolates were defined as resistant to erythromycin, and in most countries resistance rates not only exceeded 10% (using CLSI breakpoints), but also exceeded rates of penicillin resistance.[76] In China, macrolide resistance remains a common feature of *S. pneumoniae* isolates, with one study demonstrating erythromycin and azithromycin resistance rates of 96.4% and 95.8% (both using CLSI breakpoints), respectively.[77]

2.3.2 *Streptococcus pyogenes* and *Staphylococcus aureus*

Macrolide resistance is also prevalent among *S. pyogenes* isolates, with prevalence varying by country. However, prevalence has been shown to fluctuate over time in certain regions, with some reports suggesting that prevalence may have declined in recent years in some regions, even where macrolide use remained stable. Yamaguchi et al. (2015) demonstrated increasing rates of erythromycin and azithromycin resistance among community acquired *S. pyogenes* isolates in Japan over the period of 2007 to 2010, while Smit et al. (2015) observed resistance rates among a group of invasive isolates in Finland to have increased over the period of 2008 to 2013 from 1.9% to 8.7% against erythromycin and from 0.9% to 9.2% against clarithromycin (EUCAST breakpoints).[78,79] On the other hand, Silva-Costa et al. demonstrated a gradual decline in erythromycin resistance prevalence among a large collection of *S. pyogenes* isolates from Portugal from throat swabs of patients diagnosis with tonsillo-pharyngitis over the period of 1999 (20%) to 2006 (12%), with a subsequent decline over the period of 2007 (10%) to 2013 (1%) (using CLSI breakpoints).[44,80] While resistance

among pharyngeal isolates has historically been highly prevalent in Italy (Dicuonzo 2002),[81-83] resistance appears to have declined in recent years, with one study finding a relatively low rate of erythromycin resistance (7.4%) among a collection of 592 isolates from children with pharyngitis (using EUCAST breakpoints).[84] Evidence suggests that these fluctuations in overall resistance, which are also often associated with changes in phenotype (MLS_B and M phenotypes) dominance and composition, and observed variations in resistance prevalence by region may be related not only to potential changes in macrolide use but also to changes and instability in bacterial population clonality over time. A number of studies have analysed the prevalence of different *emm* type clones among populations of *S. pyogenes* and have found fluctuations in the proportions of different clones within the population alongside resistance prevalence changes, emphasizing the importance of both antibiotic consumption and population clonal structure in explaining resistance prevalence within *S. pyogenes* populations.[79,80,85]

Macrolide resistance is a well-known issue among *S. aureus* isolates. Erythromycin resistance has been detected globally among isolates and it has been demonstrated that macrolide-resistant strains can be isolated from healthy people and patients alike.[86] Abbas et al. (2015) reported an erythromycin resistance rate of 40.2% (using CLSI breakpoints) among a collection of 500 clinical *S. aureus* isolates from various body sites.[87] A number of studies have shown that macrolide resistance may be more prevalent among methicillin-resistant *S. aureus* (MRSA) isolates than methicillin-susceptible *S. aureus* (MSSA) isolates. One study performed on a collection of *S. aureus* isolates from China (where macrolide resistance is highly prevalent among respiratory isolates) demonstrated resistance rates of 82.2% among a collection of 73 MRSA isolates and 63% among a collection of 403 methicillin-susceptible *S. aureus* MSSA isolates (both using CLSI breakpoints).[77] Asbell et al. (2015) reported an azithromycin resistance rate of 63.3% (using CLSI breakpoints) among a mixed collection of

MRSA and MSSA strains containing 1169 isolates; when separated by methicillin resistance status, resistance rates were 93.3% among 493 MRSA isolates and 41.9% among 676 MSSA isolates.[88]

2.3.3 *Haemophilus influenzae*

Reported macrolide resistance rates among *H. influenzae* isolates must be interpreted with care. By nature, most wild-type strains have macrolide minimum inhibitory concentrations (MIC) that are higher than what would be observed in susceptible isolates of other species such as *S. pneumoniae*, and the majority of these strains will fall above the susceptibility breakpoints defined by EUCAST (see Figures 2.5 and 2.6) and CLSI. However, the MICs for most of these "non-susceptible" strains tend to fall only slightly above the susceptibility breakpoint, and will often fall below the resistance breakpoint (and will be defined as "intermediate"). Therefore, stating that there is a low rate of susceptibility to macrolides among any group of *H. influenzae* isolates does not necessarily mean that those non-susceptible isolates carry high level resistance to macrolides, and interpretation should always consider the MIC_{50} and MIC_{90} values of the collection where available.

Jacobs et al. reported less than 1% of a global collection of 8523 *H. influenzae* isolates were susceptible to erythromycin and clarithromycin, and less than 2% were susceptible to azithromycin (using PK/PD breakpoints).[67] However, in that particular study, clarithromycin resistance prevalence was less than 1% (using CLSI breakpoints), and susceptibility rates of 79.6% and 99.5% (both using CLSI breakpoints) were reported for clarithromycin and azithromycin, respectively. MIC_{90} values were 8 µg/mL, 16 µg/mL and 2 µg/mL for erythromycin, clarithromycin and azithromycin, respectively,[67] suggesting that the majority of the isolates were inhibited by these antibiotics at relatively low concentrations and fell within or only slightly above the intermediate range. Furthermore, 99.8% susceptibility rates

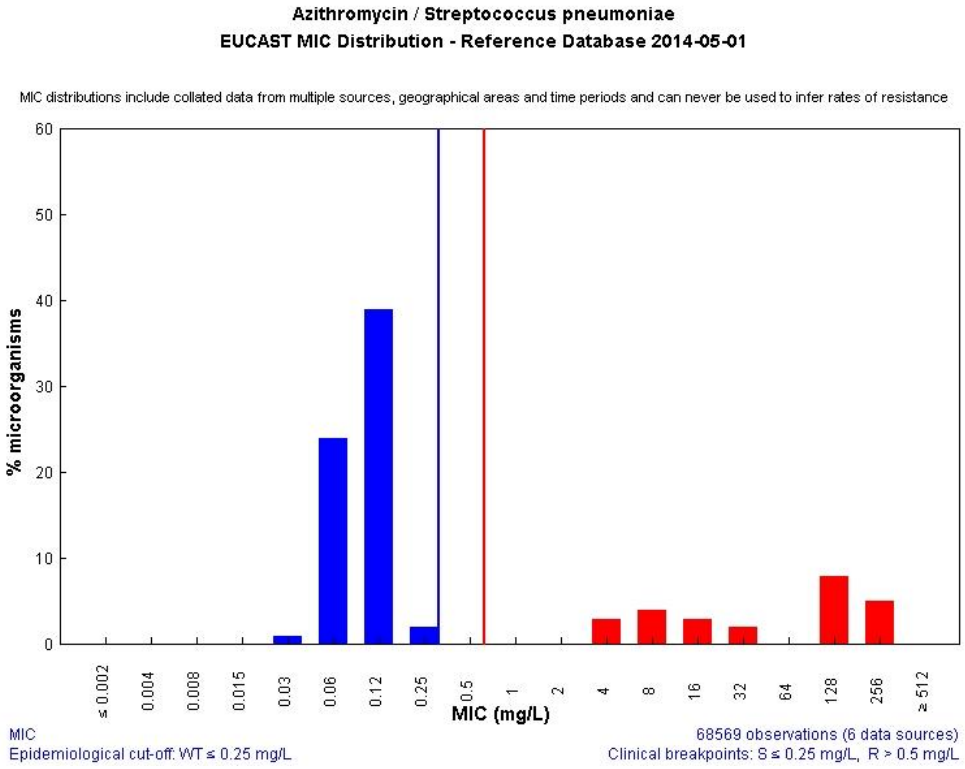

Figure 2.5: EUCAST azithromycin MIC distribution for *Streptococcus pneumoniae* (2014). Two distinct groups of isolates (susceptible and resistant) are apparent.

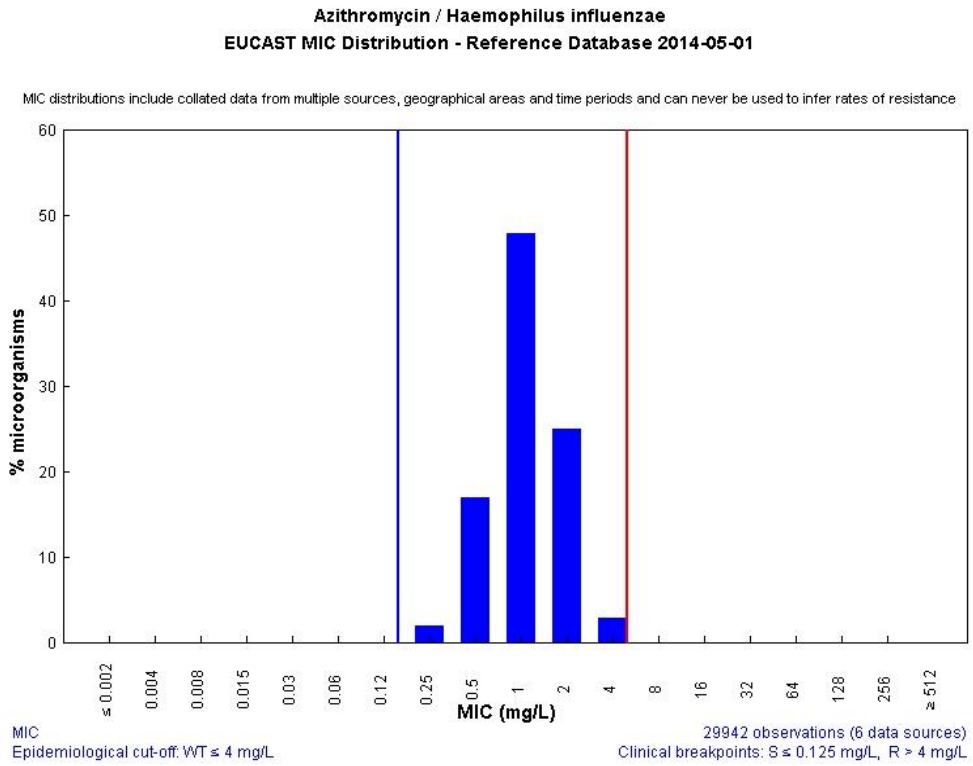

Figure 2.6: EUCAST azithromycin MIC distribution for *Haemophilus influenzae* (2014). Most isolates cluster in the intermediate range between the breakpoints.

against azithromycin and 89.4% susceptibility rates against clarithromycin (both using CLSI breakpoints) were reported for a collection of 2948 isolates cultured from various body sites via the PROTEKT study,[89] and Peric et al. (2003) demonstrated that only 1.3% of collection of 6,382 *H. influenzae* isolates had an azithromycin MIC > 4 µg/mL (used to represent "high-level resistance"), with only 2.5% having a clarithromycin MIC > 16 µg/mL.[90] A number of recent studies have repeatedly demonstrated low rates of high-level macrolide resistance in *H. influenzae*[77,88,91] even in situations where selective antibiotic pressure is present.[92] However, prevalence has been demonstrated to be higher in particular test groups: Cardines et al. (2012) observed an azithromycin resistance rate of 10.1% (using EUCAST breakpoints) in a group of 79 isolates from children with CF,[32] while Roberts et al. (2011) found 27 out of a group of 106 isolates from CF patients on an azithromycin placebo-controlled trial were resistant to erythromycin and/or azithromycin (using BSAC interpretive criteria; erythromycin MIC > 8 µg/mL; azithromycin MIC > 4 µg/mL).[93] Marchese et al. (2005) reported a clarithromycin resistance prevalence rate of 11.2% among clinical respiratory isolates collected in Italy during the year 2000 (breakpoints not described).[94] Pfaller et al. (2012), reporting on antibiotic resistance trends in the USA as part of the AWARE Ceftaroline Surveillance Program, found that azithromycin non-susceptibility was uncommon among *H. influenzae* isolates (including both respiratory and bloodstream infection isolates), but that a slight increase in resistance occurred over the period of 2008-2010 (0.8% in 2008 to 1.4% in 2010, using CLSI methodology).[75]

2.4 Overview of macrolide resistance mechanisms among key respiratory bacteria

The issues surrounding the mechanisms behind macrolide resistance are complex and expansive, and a significant body of literature has been developed that describes both acquired genes and chromosomal alterations that are associated with resistance to macrolides. This section of the chapter will provide an overview of commonly encountered macrolide resistance mechanisms among respiratory isolates, including commonly encountered macrolide resistance genes and resistance-associated chromosomal mutations.

2.4.1 Acquired macrolide resistance genes

Macrolide resistance has long been attributed to acquired genes. An online database of the currently identified MLS_B resistance genes is maintained by Dr. Marilyn Roberts;[95] this database is frequently updated and the genes listed within this database at this time are also listed in Tables 2.5, 2.6 and 2.7 of this chapter. A large number of these genes have been described but can be separated into three broad groups based on their mechanism of action. The first group describes a collection of rRNA methylase-coding genes. The genes in this group are called the *erm* genes and the methylases encoded by them act by altering the binding site of the macrolides to the ribosome to reduce macrolide binding affinity. The second group describes genes that encode various efflux pumps with action against macrolides. The *mef* genes are examples of genes contained within this group. Finally, a third group of genes encode for enzymes that act directly on the macrolide to inhibit their action. The focus of this chapter will primarily be placed on genes that are considered clinically important among macrolide-resistant respiratory isolates, namely the *erm* and *mef* groups.

2.4.1.1 *erm* and rRNA methylation

The *erm* genes were among the first genes detected that were found to provide protection against macrolides. These genes code for rRNA methyltransferases which add methyl groups to the 23S moiety of the 50S rRNA subunit, altering the binding site of the macrolide and inhibiting its actions through the prevention of efficient drug binding.[1] The methyl groups are typically described as being added specifically at position A2058 within the peptidyltransferase centre; this numbering is based on *E. coli* positioning, but has also been applied to other organisms in some instances for uniformity of the nomenclature.[96]

Roberts et al. (1999) made an attempt to standardise the *erm* nomenclature system,[97] which was revised in 2008.[1] The current system groups different *erm* genes based on their amino acid homology with previously described *erm* variants (a cut-off <80% homology is required to assign any variant as a new gene; otherwise, the gene is assigned a title based on its homology with the current groups).[1] Currently, 38 different *erm* gene types had been described and listed by Roberts (see Table 2.5).[95] The *erm* gene group is diverse and widely disseminated in Gram positive bacteria.[98] While each *erm* gene has been more strongly associated with a particular genus, many are not limited to one species and have disseminated among other species.[1] Of the many *erm* gene types, *erm*(A), *erm*(B), and *erm*(C) are among the most commonly encountered among Gram positive bacterial respiratory pathogens.

The *erm* gene group is capable of producing high-level resistance to macrolides, lincosamides and streptogramin B, all of which are structurally distinct but bind to the same site in the bacterial ribosome (Chancey 2012). This phenotype is referred to as the MLS_B resistance phenotype. Some *erm* genes do not produce resistance to the entire MLS_B group however. Expression of *erm* genes may be inducible or constitutive; bacteria with inducibly-expressed variants produce an inactive form of methylase mRNA that becomes active in the presence

Table 2.5: List of currently identified* rRNA methylation genes and the genera in which they have been detected.[95]

Gene	Genera	Gene	Genera
erm(A)	*Aggregatibacter, Bacteroides, Enterococcus, Haemophilus, Peptostreptococcus, Prevotella, Staphylococcus, Streptococcus, Helcococcus*	*erm*(30)	*Streptomyces*
erm(B)	*Aggregatibacter, Acinetobacter, Aerococcus, Arcanobacterium, Bacillus, Bacteroides, Campylobacter, Citrobacter, Corynebacterium, Clostridium, Enterobacter, Escherichia, Eubacterium, Enterococcus, Fusobacterium, Gemella, Haemophilus, Klebsiella, Lactobacillus, Micrococcus, Neisseria, Pantoeae, Pediococcus, Peptostreptococcus, Porphyromonas, Proteus, Pseudomonas, Rothia, Ruminococcus, Serratia, Shigella, Staphylococcus, Streptococcus, Treponema, Ureaplasma,*	*erm*(31)	*Streptomyces*
erm(C)	*Aeromonas, Aggregatibacter, Actinomyces, Arcanobacterium, Bacillus, Bacteriodes, Brevundimonas, Burkholderia, Chryseomonas, Clostridium, Corynebacterium, Escherichia, Eubacterium, Enterococcus Haemophilus, Lactobacillus, Macrococcus, Micrococcus, Neisseria, Paenibacillus, Pasteurella, Prevotella, Peptostreptococcus, Pseudomonas, Rhizobium, Sorhizobium, Sphingomonas, Stenotrophomans , Staphylococcus, Streptococcus, Streptomyces, Wolinella*	*erm*(32)	*Streptomyces*
erm(D)	*Bacillus, Salmonella*	*erm*(33)	*Staphylococcus*
erm(E)	*Bacteroides, Eubacterium, Fusobacterium, Ruminococcus, Saccharopolyspora, Shigella, Streptomyces*	*erm*(34)	*Bacillus*
erm(F)	*Aggregatibacter, Actinomyces, Bacteroides, Capnocytophaga, Clostridium, Corynebacterium, Eubacterium, Enterococcus, Fusobacterium, Gardnerella, Haemophilus, Lactobacillus, Mobiluncus, Neisseria, Porphyromonas, Prevotella, Peptostreptococcus, Riemerella, Ruminococcus, Shigella, Selenomonas, Staphylococcus, Streptococcus, Treponema, Veillonella, Wolinella*	*erm*(35)	*Bacteroides*
erm(G)	*Bacillus, Bacteroides, Catenibacterium, Lactobacillus, Prevotella, Porphyromonas, Staphylococcus*	*erm*(36)	*Micrococcus*
erm(H)	*Streptomyces*	*erm*(37)	*Mycobacterium*
erm(I)	*Streptomyces*	*erm*(38)	*Mycobacterium*
erm(N)	*Streptomyces*	*erm*(39)	*Mycobacterium*
erm(O)	*Streptomyces*	*erm*(40)	*Mycobacterium*
erm(Q)	*Aggregatibacter, Bacteroides, Clostridium, Staphylococcus, Streptococcus, Wolinella*	*erm*(41)	*Mycobacterium*
erm(R)	*Arthrobacter, Aeromicrobium*	*erm*(42)	*Mannheimia, Pasteurella, Photobacterium*
erm(S)	*Streptomyces*	*erm*(43)	*Staphylococcus*
erm(T)	*Enterococcus, Erysipelothrix, Haemophilus, Lactobacillus, Streptococcus, Staphylococcus*	*erm*(44)	*Staphylococcus*
erm(U)	*Streptomyces*	*erm*(44)$_v$	*Staphylococcus*
erm(V)	*Brevundimonas, Chryseomonas, Eubacterium, Fusobacterium, Leifsonia, Mesorhizobium, Paenibacillus, Pseudomonas, Rhizobium, Shewanella, Streptomyces*	*erm*(45)	*Staphylococcus*
erm(W)	*Micromonospora*	*erm*(46)	*Rhodococcus*
erm(X)	*Actinobaculum, Arcanobacterium, Bifidobacterium, Burkholderia, Brevundimonas, Corynebacterium, Leifsonia, Paenibacillus, Propionibacterium, Pseudomonas, Rhizobium, Shewanella, Sphingomonas, Stenotrophomans, Streptomyces*	*erm*(47)	*Helcococcus*
erm(Y)	*Staphylococcus*	*cfr***	*Bacillus, Escherichia, Jeotgalicoccus, Macrococcus, Staphylococcus, Proteus*
erm(Z)	*Streptomyces*	*cfr*(B)**	*Clostridium, Enterococcus*

*Current as of December 16th 2016

**The *cfr* genes are not associated with macrolide resistance but are associated with resistance against other MLS_B drugs including lincosamides.

of an inducer, while bacteria with constitutively-expressed variants produce active forms in the absence of an inducer.[99] The expression status of an *erm* gene is related to the presence and structure of an attenuator upstream of the structural gene.[99]

It is worth noting that only certain macrolides are capable of inducing expression of the *erm* genes, and that lincosamides and streptogramin B are generally not inducers of *erm*.[98] In the case of macrolides, common structural elements of inducers include a 14- or 15-membered macrolide ring, a monosaccharide at the C-5 position of the macrolide ring, and cladinose at the C-3 position.[98] It has been suggested that the C-3 cladinose is the key determinant of a macrolide being able to act as an inducer because the ketolides, generally reported to be non-inducers, have the correct ring size and the C-5 monosaccharide, but lack the C-3 cladinose. However, several ketolides including cethromycin and telithromycin have been demonstrated to be inducers of *erm*,[100] and previous reports of ketolides being non-inducers (based on their phenotypic antimicrobial activity) may be reflective of their potent antimicrobial activity irrespective of the presence of ribosomal methylation.[98]

Constitutively expressed variants tend to produce the complete MLS_B resistance phenotype.[101] The resistance phenotype produced by inducible *erm* appears to be dependent on the specific *erm* gene variant, with some variants producing a variety of phenotypes in different isolates and species, and others producing a more predictable phenotype.[99] In inducible variants, resistance to 16-membered macrolides, lincosamides and streptogramin B may not be present unless expression of the genes has been induced by 14- or 15-membered macrolides like erythromycin and azithromycin,[98] but the use of non-inducing macrolides and other MLS_B antibiotics in treating infections with bacteria carrying inducibly-expressed variants of *erm* still carries significant risk. Mutants with constitutively-expressed variants can be selected easily *in vitro*, and this has also been reported in patients undergoing clindamycin therapy against inducibly-resistant *S. aureus*.[99]

While *erm* is the primary gene group that encodes rRNA methylase-mediated MLS_B resistance, the *cfr* genes have also been identified as encoders of a similar mechanism. These genes are associated with resistance against lincosamides and streptogramin A, but unlike the *erm* genes they are not associated with macrolide resistance. One of these genes, simply referred to as *cfr*, has been identified in 6 genera including *Staphylococcus*, *Bacillus* and *Escherichia*. This gene causes methylation at position A2503 of 23S rRNA that results in clindamycin and chloramphenicol resistance and has been detected on the plasmid pSCFS3. *cfr*(B) is chromosomally located and has been reported in the genera *Clostridium* and *Enterococcus*.[95]

2.4.1.1.1 *erm*(A)

The *erm*(A) gene has long been associated with *S. aureus*, particularly with MRSA,[99] and has historically been widespread among staphylococci.[102] *erm*(A) has been reported among at least nine different genera, including *S. pyogenes*[103] and *Enterococcus* spp.,[104] although it has not disseminated as widely as *erm*(B) or *erm*(C).[95] Mazzariol et al. (2007) reported a 73.9% prevalence rate of *erm*(A) among a group of 69 *S. pyogenes* with an $iMLS_B$ phenotype. On the other hand, the same group did no detect *erm*(A) among a group of 40 *S. pyogenes* with a $cMLS_B$ phenotype, nor did they find any incidence of *erm*(A) among a collection of 145 erythromycin-resistant *S. pneumoniae* isolate regardless of whether they had an $iMLS_B$ phenotype or a $cMLS_B$.[103] Jensen et al. (1999) found that among a collection of 44 erythromycin-resistant isolates of *S. aureus* of human origin, 10 were found to carry *erm*(A), three of which also carried *erm*(C). The same group did not detect *erm*(A) among a collection of 17 *Enterococcus faecium* isolates of human origin.[105]

Sequencing of *erm*(A) revealed that the gene codes for a 243-amino acid protein that is homologous to that produced by other *erm* variants, including *erm*(C). Murphy (1985) reported that *erm*(A) was less closely related to *erm*(B).[106] *erm*(A) is regulated by

translational attenuation, and is preceded by an attenuator structure that encodes two leader peptides.[98] The *erm*(A) attenuator model in *S. aureus* has been studied and in this particular allele, the presence of an inducer results in stalled ribosome complexes forming on the cistrons of the leader peptides *erm*(A)L1 and *erm*(A)L2. Stalling of *erm*(A)L1 results in translation of *erm*(A)L2, and stalling on *erm*(A)L2 allows for translation of the *erm*(A) gene.[107] However, mutations that disrupt the attenuator structure can result in constitutive expression of *erm*(A) in some Gram positive species, including *S. aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae*.[98,108]

2.4.1.1.2 Mobile genetic elements carrying *erm*(A)

The chromosomally-located 6691-bp element Tn*554*, detected in *S. aureus*, was among the earliest genetic elements described that was shown to carry *erm*(A).[106] Tn*554* is associated with inducible MLS_B resistance and is integrated into the *S. aureus* chromosome at a particular insertion site, *att554*.[109,110] Tn*554* will almost always be inserted in a particular orientation.[110] This element consists of 6 ORFs. Three ORFs represent genes encoding functions essential for transposition, *tnpA*, *tnpB*, and *tnpC*. *tnpA* and *tnpB* are thought to encode products that catalyze the recombination reaction while *tnpC* controls the efficiency of the reaction and determines the orientation of Tn*544* in *att554* (mutations in *tnpC* can result in Tn*544* being inserted in the opposite direction of the chromosome).[111] Two other ORFs were identified as *spc* (encoding resistance to spectinomycin) and *erm*(A).[110]

Tn*554* is widespread among isolates and is the best described *erm*(A)-carrying element, although composite elements with homology to Tn*554* have also been detected. For example, Tn*6133*, described in MRSA, is an 11,475-bp transposon that was found to consist of Tn*554* with a 4-787-bp insertion, in which the streptogramin A-, pleuromutilin-, and lincosamide-resistance-encoding gene *vga(E)* was contained.[109]

2.4.1.1.3 *erm*(B)

erm(B) was described in *Streptococcus sanguinis* (formerly *Streptococcus sanguis*) as early as 1978.[112] The gene has since spread across many species worldwide[113] and has been detected in more individual genera than any other *erm* gene currently listed in the Roberts database.[95] In 2008, *erm*(B) was reported to be the most common macrolide resistance mechanism among erythromycin resistant *S. pneumoniae* isolates (55.0% globally)[114] in most countries[76] and particularly widespread among streptococcal species.[98,115] Studies have continued to a report high incidence of *erm*(B) among macrolide-resistant clinical isolates of *S. pneumoniae*, often in the presence of *mef*. Azadegan et al. (2015) reported an *erm*(B) prevalence rate of approximately 84% among a collection of 88 erythromycin- and clarithromycin-resistant clinical and normal flora *S. pneumoniae* isolates from Iran; all *erm*(B) carrying isolates were associated with a $cMLS_B$ phenotype, and 40% of the 88 resistant isolates carried *erm*(B) and *mef*.[116] Kohno et al. (2014) reported *erm*(B) in six out of eleven azithromycin-resistant clinical *S. pneumoniae* isolates from Japan, with 2 of these isolates also carrying *mef*(A).[117] Mazzariol et al. (2007) reported that among a collection of 119 erythromycin-resistant clinical isolates *S. pneumoniae* exhibiting an MLS_B phenotype, all isolates carried *erm*(B) regardless of whether the phenotype was constitutively or inducibly expressed. In addition, the same group also reported that among a collection of 40 *S. pyogenes* with a $cMLS_B$ phenotype, all carried *erm*(B), and among a collection of 69 *S. pyogenes* with an $iMLS_B$ phenotype, 26.1% carried *erm*(B).[103] Jensen et al. (1999) reported that 100% of a group of 17 erythromycin-resistant *E. faecium* isolates of human origin carried *erm*(B), and did not detect any instances of *erm*(B) among a collection of 44 erythromycin-resistant isolates of *S. aureus* of human origin.[105]

The protein produced by *erm*(B) as reported by Horinouchi et al. (1983) was predicted to be 246 amino acids in length; the same group stated that *erm*(B) likely shares a common origin

with other *erm* genes due to their sequence homology.[118] The mechanism of *erm*(B) induction has not been as thoroughly studied as it has with *erm*(C). Much like *erm*(A), *erm*(B) induction is also regulated by translational attenuation with stalling of *erm*(B) expression, but in this case there is only a single leader peptide sequence with no resemblance to that of *erm*(A) or *erm*(C).[98] Expression of *erm*(B) can be inducible or constitutive; mutations in the leader peptide sequences have been demonstrated to allow for constitutive expression of *erm*(B) in *S. pneumoniae, S. pyogenes* and *S. agalactiae*.[98,103,108,119] Interestingly, the phenotype produced in bacteria by inducibly expressed variants of *erm*(B) can vary greatly compared to inducibly expressed variants of *erm*(A) or *erm*(C), which tend to fit the expected phenotypic profile of resistance to 14- and 15-membered macrolides but no resistance to 16-membered macrolides.[99]

2.4.1.1.4 Mobile genetic elements carrying *erm*(B)

The group of elements bearing *erm*(B) is more diverse than what is seen with *erm*(A). Among the earliest reported elements was pAM77, a plasmid isolated from an isolate of *S. sanguinis* with the MLS_B phenotype and derived from dental plaque of a patient who had been on long-term erythromycin therapy.[112] This plasmid, as well as the *erm*(B)-carrying plasmid pAMβ-1, has also been detected in *E. faecalis*. Other plasmids capable of carrying *erm*(B) that have been described include pTE80 in *Lactobacillus reuteri*, pBT233 in *Bacillus subtilis*, pMD101 in *S. pyogenes*, pIP501 in *S. agalactiae*, pLEM3 in *Lactobacillus fermentum*, pIP1527 in *E.coli*, and pIP402 in *Clostridium perfringens*.[95,120-125]

Among the most studied *erm*(B)-carrying elements is Tn*917*, first described in *Enterococcus faecalis*,[126,127] and originally described on a multiresistance plasmid, pAD2, with no conjugative properties.[128] Tn*917* has since been shown to be incorporated into a variety of other conjugative elements.[115,129] Sequencing of Tn*917* revealed that the transposon contains 5 ORFs, including *erm*(B) and the transposition-related genes *tnpR* and *tnpA*.[115] Subsequent

to the description of Tn*917*, structures with similar sequences were described in *S. pneumoniae*. Tn*3872*, a conjugative element,[115, 133] is the result of the insertion of Tn*917* into *orf9* of the *tet*(M)-carrying Tn*916*, creating a linkage between *erm*(B) and *tet*(M).[130,131] Elements similar in structure to Tn*3872* have since been found in *S. pyogenes*; Tn*917* was also found in other isolates and may have been present on alternate conjugative elements.[115,132] A composite element corresponding to Tn*3872* has also been described in *S. agalactiae*.[133]

Elements with Tn*916*-like features are important mediators of resistance among streptococci,[134] and a number of other *erm*(B)-bearing transposons containing a Tn*916*-like element with a *erm*(B)/*tet*(M) linkage have been described.[115] In some of these elements, the *erm*(B)-carrying element is a 2,874bp element consisting of 5 ORFs from *orfP0* to *orfP4* (*erm*(B) is *orfP2*, the third ORF), and inserts into a different position of Tn*916* than is seen in Tn*3872* (position 3847 of published sequenced).[115] In Tn*6002*, the *erm*(B) element is inserted alone. This conjugative element[133, 135] has been reported in *S. pyogenes*[132] and *S. pneumoniae*.[132,135-137] The conjugative[136] Tn*6003* can be distinguished from Tn*6002* by the additional insertion of the MAS (macrolide-aminoglycoside-streptothricin) element, inserted between *orfP0* and *orfP1* within the *erm*(B) element.[115] The insertion of MAS, which contains an additional *erm*(B) gene and its leader peptide, into Tn*6003* may be related to a 222-bp sequence, normally absent in Tn*916*, found on the left end of the *erm*(B) element and on the right end of the MAS element.[136] Tn*6003* is less commonly encountered than Tn*6002*, possibly due to the instability of the MAS element.[138] Tn*2010* is another *erm*(B)-carrying Tn*916*-like transposon that has been described in *S. pneumoniae*. The element contains an insertion of a variant of mega (macrolide efflux genetic assembly), a *mef*(E)-carrying element, at position 17014 of the Tn*6002* sequence. It does not appear to be transferable by conjugation.[137,139]

The approximately 50kb element Tn*1116* has been described in *S. pyogenes*.[132] Tn*1116* is the result of an insertion of an *erm*(B)-carrying element into the coding sequence of *tet*(M) within a defective variant of Tn*5397*, a Tn*916*-related, *tet*(M)-carrying element originally described in *Clostridium difficile*, in which the *xis* (excisase) and *int* (integrase) genes typical of Tn*5397* are replaced with the *tndX* (resolvase) gene.[140] The involvement of Tn*5397* and the resulting truncated and silent[140] *tet*(M) distinguishes Tn*1116* from other Tn*916*-like elements. Tn*1116* has been shown to demonstrate homology with the *S. pyogenes* plasmid pSM19035[115,121] and is easily transferred in intraspecific matings.[132]

In *S. aureus*, *erm*(B) has been reported on the transposon Tn*551*.[141,142] This approximately 5.2kb element was detected on a set of plasmids derived from Japan in the 1960s that shared the same prototype, pI258, and isolates carrying this resistance determinant were shown to exhibit a $cMLS_B$ phenotype.[110,142] This transposon encodes five putative proteins and has been reported to be extremely similar to Tn*917* in terms of genetic organisation and nucleotide sequence, with the two transposons differing at just 11 positions along the entire sequence.[143]

2.4.1.1.5 *erm*(C)

erm(C) is among the most widely disseminated and clinically important determinants of the MLS_B phenotype in Gram positive organisms. *erm*(C) has been identified in at least 32 different genera.[95] *erm*(C) is particularly prevalent among staphylococci,[1,98,101] and while *erm*(A) was historically considered the primary *erm* gene in *S. aureus*, *erm*(C) prevalence has been shown to exceed that of *erm*(A) in certain contexts. Eady et al. (1993) reported that *erm*(C) was the predominant *erm* gene among a highly-diverse collection of erythromycin-resistant *Staphylococcus* spp., which *erm*(C) prevalence at 51.2% among the human isolates included in the study. By comparison, *erm*(A) prevalence was 8.1% among the same group of isolates and no instances of *erm*(B) were reported in the study.[144] In addition, Westh et al.

(1995) found that *erm*(C) prevalence had risen among a collection of blood-derived erythromycin-resistant *S. aureus* isolates from Denmark collected over the period of 1959-1988, being absent prior to 1971 but becoming the predominant *erm* gene from 1984 onwards. Prior to the detection of *erm*(C), *erm*(A) had been the predominant *erm* gene.[102] In addition, Jensen et al. (1999) detected 36 instances of *erm*(C) among a collection of 44 erythromycin-resistant isolates of *S. aureus* of human origin, compared to 10 instances of *erm*(A) in the same group of isolates (three of the isolates carried both *erm*(A) and *erm*(C)).[105] *erm*(C) is not exclusive to staphylococci however, and the gene has also been reported in *Streptococcus* spp., *Enterococcus* spp. and a number of anaerobic species including *Bacteroides* spp..[95]

The product of *erm*(C) is 244 amino acids in length.[145] *erm*(C) can be constitutively or inducibly expressed depending on the presence of a complete attenuator.[144] The *erm*(C) induction model is the best described model of *erm* induction. It is known that *erm*(C) can be induced by 14- and 15-membered macrolides, and that expression is controlled by translational attenuation.[98,101] Expression of *erm*(C) is also dependent on the presence and specific structure of the leader sequence,[101,146,147] which contains a small open reading frame that encodes ErmCL, a 19-amino acid leader peptide.[98] This leader sequence is separated from the structural gene by a 60-bp spacer region containing 2 pairs of inverted repeats rich in G/C content. These inverted repeats are involved in the folding of the leader sequence in different conformations during both non-inducing and inducing conditions.[98] When the inducer is absent, the first and second inverted repeats anneal with each other to form stem-loop 1+2, while the third and fourth repeats anneal with each other to form stem-loop 3+4. The formation of stem-loop 3+4 sequesters the ribosomal binding site of *erm*(C) (RBS_2), blocking the translation of the gene.[98] When an inducer is introduced, the structure of the

stem-loops is disrupted and the second and third inverted repeats are allowed to anneal and form stem-loop 2+3. This frees RBS_2 and allows for *erm*(C) translation to occur.

The formation of the active or inactive conformation is dependent on the translational status of the ribosome on the *erm*(C)L leader cistron.[98] In the absence of an inducer, the ribosome unwinds stem-loop 1+2, thereby translating *erm*(C)L and dissociating from the transcript. The inactive conformation then forms via the refolding of stem-loop 1+2.[98,148] When an inducer is bound to the nascent peptide exit tunnel (NPET) of the 50S ribosomal subunit, the ribosomal complex is stalled on *erm*(C)L. This prevents the reformation of stem-loop 1+2 and allows for *erm*(C) to be translated.[98] Stalling of the ribosome on *erm*(C)L depends on how the proteins carrying the nascent peptide interact with ribosomal nucleotides as it travels through the NPET;[149] the specific position of stalling in the presence of an inducer is dictated by the interactions between nucleotides near the peptidyl transferase center and carboxyl terminus of the nascent leader peptide.[98]

2.4.1.1.6 Mobile genetic elements carrying *erm*(C)

In staphylococci, a large number of small multi-copy plasmids carrying *erm*(C), typically ranging from 2.2-2.5kb in size but with a number of exceptions, have been described. Many of these plasmids are capable of mobilization between different staphylococcal species, and also between staphylococcal species and *B. subtilis*.[101] In *S. aureus*, the plasmid pE194 was among the earliest described plasmids capable of carrying *erm*(C).[147,150,151] The nucleotide sequence of pE194, which contains 3728bp, was first deduced by Horinouchi et al. (1982), who were able to localize the determinants for MLS_B resistance and plasmid replication within pE194 using site-specific cleavage with restriction enzymes. The *erm*(C) gene was reported to be constitutively expressed in this case.[152] A number of other plasmids carrying both inducible and constitutively expressed variants of *erm*(C) plasmids have since been identified. Many of these plasmids carry resistance regions homologous to pE194 and likely

share a common evolutionary origin, although the replication regions of these plasmids may differ from pE194.[110]

The naturally-occurring 2475bp plasmid pT48 of *S. aureus* confers the MLS_B phenotype and was completely sequenced in 1988. It was found to contain a region homologous to pE194 that contained *erm*(C) and the leader peptide that allows for inducible expression.[141] By contrast, the *erm*(C) gene found on the closely related plasmid pNE131 is constitutively expressed due to a deletion of the leader sequence that regulates translational attenuation of the gene.[141] Constitutively expressed *erm*(C) variants in *S. aureus* have also been described on pJ74 and pA22 (2.5kb), attributable to leader sequence deletions,[141,153] and on the 2.55kb pRJ5, attributable to a duplication within the leader region (Oliviera 1996). *erm*(C)-carrying plasmids have also been detected in other staphylococci. Examples include pPV141 found in *Staphylococcus chromogenes*[154] and pPV142 found in *Staphylococcus simulans* (2.5kb, constitutively expressed, shares homology with pPV141),[155] A variety of *erm*(C)-carrying plasmids identified in staphylococci derived from animal sources have also been described, including pSES4a from *Staphylococcus haemolyticus* (2.3kb, constitutively expressed), pSES5 from *Staphylococcus hominis* (2.4kb, inducibly expressed), and pSES6 from *Staphylococcus equorum* (4.0kb, constitutively expressed).[95,101] The *erm*(C) variants on each of 3 animal-sourced plasmids listed above exhibited high degrees of homology with that of pE194, and the methylase produced by the *erm*(C) on pSES6 was indistinguishable from that of pE194.[101]

In other species outside of staphylococci, other *erm*(C)-carrying plasmids have also been described. Another naturally occurring *erm*(C)-carrying plasmid, pIM13, has been described in *B. subtilis*.[156,157] The plasmid is a close relative to pE5, an inducible *erm*(C)-carrying plasmid that is native to *S. aureus*, and the *erm*(C) resistance determinant was found to be greater than 90% homologous to that of pE194.[157] The *erm*(C) gene found on pIM13, which

likely arose from pE5, was constitutively expressed due to a deletion in the leader sequence that is present on pE5.[157]

2.4.1.1.7 Other *erm* genes

As mentioned previously, a large number of *erm* gene types have been described,[95] although most of these genes have not been reported among *Staphylococcus* spp. and *Streptococcus* spp.. *erm*(F), *erm*(Q) and *erm*(T) have been reported among *Streptococcus* spp.,[95,97,158,159] while *erm*(F), *erm*(G), *erm*(Q), *erm*(T), *erm*(Y), *erm*(42), *erm*(43) and *erm*(44) have been reported among *Staphylococcus* spp..[1,95,158,160,161] An *erm*(A)/*erm*(C) recombination product designated *erm*(33) has also been reported in *Staphylococcus sciuri*.[162] *erm*(T) has been detected in inducibly resistant invasive *S. pyogenes* isolates, group D streptococci, *E faecium* and livestock-associated MRSA.[159,163,164] While the *erm*(T)-carrying element has been shown to be chromosomal in nature in group D streptococci, it was shown to be carried on a 4,962 bp plasmid referred to as pRW35 in invasive macrolide-resistant *S. pyogenes* isolates.[159] In livestock-associated MRSA, the plasmid pKKS25 carries a constitutively expressed variant of *erm*(T); the *erm*(T)-bearing region of this plasmid shares >99% homology with that of pRW35.[164] A number of other plasmids have also been described as carriers of *erm*(T) in *Lactobacillus* spp.,[165,166] in which *erm*(T) was first reported. *erm*(T)-carrying elements have been shown to be mobile from *S. agalactiae* donors to *S. agalactiae* and enterococcal recipients.[167] *erm*(Y), a variant with around 80% nucleotide homology with *erm*(T) and leader peptide similarities with *erm*(C), has been detected among *S. aureus* clinical isolates on the plasmid pMS97 which also harbours the macrolide resistance genes *msr*(A) and *mph*(C).[161] *erm*(F) is also found in human pathogenic bacteria and has been reported among 25 different genera.[95] The gene is commonly associated with *Bacteroides* spp. and other anaerobic species,[99] but has been reported among clinical isolates of alpha-haemolytic streptococci, *S. agalactiae*, *S. aureus* and other *Staphylococcus* spp..[1,97,158] In *Bacteroides*

fragilis, *erm*(F) has been detected on the plasmid pBF4,[168] and transposons such as Tn*4351*[169] and Tn*4551*.[170]

An *erm*(A) subclass variant designated *erm*(TR) and associated with macrolide resistance in streptococci has been described[171] and should be noted here. This gene, reportedly inducibly expressed[172] but also reported in constitutively resistant isolates alongside other macrolide resistance genes,[173] has been shown to be widely distributed among *S. pyogenes*. One report on a global collection of erythromycin-resistant *S. pyogenes* isolates demonstrated an 11.3% prevalence rate of *erm*(TR),[174] while another study reported an *erm*(TR) prevalence rate of 30% among a collection of 60 erythromycin-resistant isolates with an $iMLS_B$ phenotype (no instances of *erm*(TR) were reported among a collection of 20 isolates with a $cMLS_B$ phenotype.[175] *erm*(TR) has also been reported among alternate beta-haemolytic *Streptococcus* spp.[176,177] as well as less frequently in *S. pneumoniae*.[178] *erm*(TR) has been shown to be carried on a number of homologous conjugative elements via mating studies using *S. pyogenes* and other gram positive organisms as recipients.[115] In *S. pyogenes*, *erm*(TR) has been demonstrated to be carried on ICE 10750-RD.2, a 49-kb integrative conjugative element (ICE) that is integrated into DNA restriction-modification methyltransferases-encoding *hsdM*.[179] In *S. pneumoniae*, *erm*(TR) is carried on Tn*1806*.[178] Tn*1806* is slightly larger in base length than ICE 10750-RD.2 but the two elements share significant homology, and Tn*1806* is integrated in a site homologous to spr0790 and corresponding to *hsdM*.[115,178]

2.4.1.2 *mef* and macrolide efflux

Macrolide resistance has also been attributed to genes encoding efflux mechanisms (see Table 2.6). Inducible efflux-mediated resistance was first observed in *S. epidermidis* and was attributed to an incomplete ABC transporter encoded by *msr*(A).[98,180] A number of other

Table 2.6: List of currently identified* MLS_B efflux-encoding genes and the genera in which they have been detected.[95]

Gene	Efflux superfamily	Resistance Profile	Genera	Gene	Efflux superfamily	Resistance Profile	Genera
mef(A)*	Major Facilitator	Erythromycin	*Acinetobacter, Bacteroides, Citrobacter, Clostridium, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Fusobacterium, Gemella, Haemophilus, Klebsiella, Lactobacillus, Micrococcus, Morganella, Neisseria, Pantoeaae, Pediococcus, Providencia, Proteus, Ralstonia, Rothia, Pseudomonas, Salmonella, Serratia, Staphylococcus, Streptococcus, Stenotrophomonas,*	*vga*(A)	ATP-binding transporter	Streptogramin A and Pleuromutilin	*Leifsonia, Paenibacillus, Staphylococcus*
mef(B)*	Major Facilitator	Erythromycin	*Escherichia*	*vga*(A)$_{LC}$	ATP-binding transporter	Streptogramin A and Lincosamides	*Staphylococcus*
mef(C)*	Major Facilitator	Erythromycin	*Photobacterium, Vibrio*	*vga*(B)	ATP-binding transporter	Streptogramin A	*Enterococcus, Staphylococcus*
lmr(A)	Major Facilitator	Lincomycin	*Streptomyces*	*vga*(C)	ATP-binding transporter	Streptogramin A and Pleuromutilin	*Staphylococcus*
car(A)	ATP-binding transporter	Carbomycin	*Streptomyces*	*vga*(D)	ATP-binding transporter	Streptogramin A	*Enterococcus*
msr(A)	ATP-binding transporter	Erythromycin and Streptogramin B	*Brevundimonas, Burkholderia, Chryseomonas, Corynebacterium, Enterobacter, Enterococcus, Gemella, Lysinibacillus, Photobacterium, Pseudomonas, Shewanella, Staphylococcus, Streptococcus, Streptomyces, Ureaplasma*	*vga*(E)	ATP-binding transporter	Streptogramin A and Pleuromutilin	*Staphylococcus*
msr(C)	ATP-binding transporter	Erythromycin and Streptogramin B	*Enterococcus*	*optr*	ATP-binding transporter	Oxazolidinones and Florfenicol	*Enterococcus*
msr(D)	ATP-binding transporter	Erythromycin and Streptogramin B	*Acinetobacter, Bacteroides, Citrobacter, Clostridium, Corynebacterium, Enterococcus, Enterobacter, Escherichia, Gemella, Fusobacterium, Haemophilus, Klebsiella, Morganella, Neisseria, Proteus, Providencia, Pseudomonas, Ralstonia, Staphylococcus, Streptococcus, Serratia,*	*eat*(V)$_V$	ATP-binding transporter	Lincomycin, Streptogramin A and Pleuromutilin	*Enterococcus*
msr(E)	ATP-binding transporter	Erythromycin and Streptogramin B	*Acinetobacter, Citrobacter, Escherichia, Klebsiella, Pasteurella, Serratia*	*sal*(A)	ATP-binding transporter	Lincomycin and Streptogramin A	*Staphylococcus*
lsa(A)	ATP-binding transporter	Lincomycin	*Enterococcus,*	*ole*(B)	ATP-binding transporter	Oleandomycin	*Lysinibacillus, Sorhizobium, Streptomyces*
lsa(B)	ATP-binding transporter	Lincomycin	*Staphylococcus*	*ole*(C)	ATP-binding transporter	Oleandomycin	*Streptomyces*
lsa(C)	ATP-binding transporter	Lincomycin	*Gardnerella, Streptococcus*	*srm*(B)	ATP-binding transporter	Spiramycin	*Streptomyces*
lsa(E)	ATP-binding transporter	Lincomycin, Streptogramin A and Pleuromutilin	*Enterococcus, Erysipelothrix, Staphylococcus*	*tlr*(C)	ATP-binding transporter	Tylocin	*Streptomyces*
lsa(E)$_V$	ATP-binding transporter	Lincomycin, Streptogramin A and Pleuromutilin	*Enterococcus, Staphylococcus*				

*Current as of December 16th 2016

**A number of other *mef* genes have been described in the literature, including *mef*(E) and *mef*(I), but these are not including within Roberts' online database.

genes encoding macrolide efflux resistance mechanisms have since been described, including the *mef* gene. The *mef* gene is predicted to encode a major facilitator family efflux pump.[98] Macrolide efflux encoded by the *mef* gene group is associated with low-level resistance, and typically with the M phenotype rather than the MLS_B phenotype associated with *erm*-mediated resistance (i.e. resistance to 14- and 15- membered macrolides but not to 16-membered macrolides, lincosamides and streptogramin B). At least 6 *mef* genes have been described,[95,115] with *mef*(A) and *mef*(E) being the earliest *mef* genes reported.[181,182]

A gene homologous to *msr*(A), an ATP-binding cassette-encoding gene associated with macrolide efflux in staphylococci,[183] is frequently reported downstream of *mef*. This gene is referred to as *msr*(D), but other names have historically been used to describe *msr*(D) depending on the *mef* gene variant (such as *mel*). Studies have demonstrated that *mef* and *msr*(D) are co-transcribed and may act as a dual efflux system,[184] inducible by erythromycin.[185] Notably, it has been reported that *msr*(D) may be more important than *mef* in producing the M-phenotype,[186] and a recent study found that *msr*(D) can almost compensate for *mef*(A) function in *S. pyogenes* when it has been knocked out, while *mef*(A) is unable to do the same for *msr*(D), indicating that of the two genes in the efflux system, in *S. pyogenes* at least, *msr*(D) may be the predominant determinant of macrolide efflux rather than *mef*(A).[187]

2.4.1.2.1 *mef* classification and nomenclature

Historically, some controversy has existed regarding the classification of the separate *mef* genes. The reported *mef* variants exhibit significant DNA and protein sequence homology to one another, with *mef*(A) and *mef*(E) exhibiting >90% protein sequence homology,[185,188] and some studies have shown that there is little difference in the phenotype produced by these 2 genes.[103] As a result, Roberts et al. (1999) put forth the argument that the separate *mef* genes types are in fact variants of the same gene, and that all of them should simply be referred to

as *mef*(A). If it were necessary to distinguish the separate gene types, the gene would be referred to as a subtype of *mef*(A) (e.g. *mef*(A) subtype *mef*(E)).[97] Currently, Roberts' online database lists three separate mef gene types: *mef*(A), *mef*(B) and *mef*(C) (see Table 2.6).[95]

Although many previously assumed that *mef*(A) and *mef*(E) were species specific, studies soon emerged where *mef*(A) was detected in community-acquired isolates *S. pneumoniae* after it was originally detected and described in *S. pyogenes*.[189,190] In recent years it has been shown that the various *mef* genes have disseminated in markedly different ways via different mobile elements, and often in association with different resistance markers,[191] and that there may even be differences in the activities of these genes in certain contexts. For example, one study looking at *mef*(A) in *S. pneumoniae* found that isolates containing *mef*(A) had consistently higher minimum inhibitory concentrations (MICs) that isolates with *mef*(E).[192] Furthermore, the 2 genes have disemminated into different species over time; while both genes had been identified in 5 species of streptococci by 2005, *mef*(E) had also been identified in numerous streptococcal, staphylococcal, and enterococcal species in which *mef*(A) had not yet been described.[193] These differences have lead to many researchers to continue to differentiate *mef*(A), *mef*(E) and the other *mef* genes, despite the recommendations of Roberts et al..[193] For the purposes of this review, *mef*(A) and *mef*(E) will be considered separate genes.

As a result of the confusion regarding *mef* nomenclature, care must be taken when interpreting prevalence data reported by surveillance studies on the different *mef* genes. In some cases, these studies will report the prevalence of "*mef*(A)" within a particular group of isolates but it is not always clear if these studies are reporting only this particular *mef* variant or have simply grouped multiple *mef* variants together as "*mef*(A)" in accordance with the recommendations of Roberts.

2.4.1.2.2 *mef*(A)

The *mef*(A) gene is among the most commonly encountered *mef* genes in macrolide-resistant Gram positive organisms and was first described by Clancy et al. (1996) in *S. pyogenes*.[181] It has since been identified in a number of other *Streptococcus* spp., including *S. pneumoniae*, *S. agalactiae* and *Streptococcus suis*.[193-195] With the increasing reliance on macrolide drugs over time, the number of isolates that have acquired this gene has increased. In a 2008 study, *mef*(A) was reported as the second most common macrolide resistance mechanism among erythromycin resistant *S. pneumoniae* isolates after *erm*(B)[114] although surveillance studies have also demonstrated that the prevalence of *mef*(A) may exceed that of *erm*(B) in certain countries, including Canada, USA and Greece.[196] Bley et al. (2011) also found that *mef*(A) was the most common macrolide resistance determinant among *S. pneumoniae* (57.7% among 437 macrolide resistant invasive and non-invasive isolates from Germany) and the second most common macrolide resistance determinant (after *erm*(B)) among *S. pyogenes* (31.0% among 29 macrolide resistant invasive and non-invasive isolates from Germany).[194] Silva-Costa et al. detected *mef*(A) in 31.7% of a collection of 161 *S. pyogenes* isolates exhibiting the M-phenotype collected over the period of 2007-2013; 49.4% of a similar collection of 156 isolates collected over the period of 2004-2006 had been previously reported.[44,80] Mazzariol et al. (2007) reported that 100% of a collection of 40 clinical isolates of *S. pyogenes* exhibiting the M phenotype carried *mef*(A), and that 69.2% of a collection of 26 clinical isolates of *S. pneumoniae* exhibiting a similar phenotype also carried *mef*(A).[103]

The *mef*(A)/*msr*(D) operon is inducible in *S. pyogenes*. While the mechanism has not been widely reported, the mechanism for the homologous system *mef*(E)/*msr*(D) has been reported[98] and it is likely that *mef*(A)/*msr*(D) is controlled by a transcriptional attenuator mechanism in a similar fashion.

2.4.1.2.3 Mobile genetic elements carrying *mef*(A)

Tn*1207.1* was among the earliest *mef*(A)-carrying elements to be described and was originally described in *S. pneumoniae*. It is a chromosomal element (integrated into *celB* of the pneumococcal chromosome) and was considered to be a defective element due to the truncated appearance of the 5' end of *orf8*.[197] Of the 8 ORFs, *mef*(A) was the fourth and *msr*(D) was the fifth. *Orf2* was homologous to site-specific recombinases of Gram positive bacteria, while *orf6*, *orf7*, and *orf8* were homologous to 3 ORFs of Tn*5252*, a conjugative transposon found in pneumococci.

In *S. pyogenes*, Tn*1207.1* is found as part of larger prophages, and it is currently understood that phage transfer plays a critical role in the dissemination of *mef*(A) in *S. pyogenes*.[198] In recent years, evidence has suggested that transduction plays a role in the horizontal transfer of these prophages.[115,199] The 52,491-bp prophage ϕ1207.3, in which Tn*1207.1* forms the left end, was originally referred to as a conjugative transposon (Tn*1207.3*) before its true nature as a prophage was elucidated[200] and has been shown to be transferable between streptococcal species, including *S. pyogenes*, *S. pneumoniae* and *Streptococcus gordonii*.[200,201] The element contains 58 ORFs, including the ORFs of Tn*1207.1*. ϕ1207.3 integrates at a single site within the bacterial chromosome of *S. pyogenes*, namely within the putative competence protein-encoding *comEC* (for which *celB* is the pneumococcal equivalent).[200] ϕ1207.3 shares significant sequence homology with the 58,761-bp ϕ10394.4, another prophage found in *S. pyogenes* in which Tn1207.1 is integrated.[200,202,203] ϕ1207.3 is identical to the right hand side of ϕ10394.4, the latter of which is also integrated into *comEC*, but ϕ10394.4 carries an additional left hand region approximately 6kb in length.[199,203] Of the 2 elements, ϕ1207.3 is the more common among *S. pyogenes* isolates carrying the tetracycline-susceptible M-phenotype.[204-206] In addition, ϕ1207.3 has been detected in *S. agalactiae*,[177] while ϕ10394.4 has been detected in *S. gordonii* (viridans variants) and *Streptococcus salivarius*.[207]

In addition, alternate *mef*(A)-carrying elements in which tetracycline resistance determinant *tet*(O) is also carried have also been described in *S. pyogenes*.[198] The most extensively described of these elements is the ~60 kb ϕm46.1,[115,199] which is not integrated into the same chromosomal site as ϕ1207.3 and ϕ10394.4 but is instead integrated into *rum*, a coding region for an RNA uracil transferase.[200,204] Particular regions of ϕ1207.3 outside of the Tn*1207.1* segment share significant homology (>70%) with ϕm46.1, although overall the homology is not shared to the same degree as that with ϕ10394.4.[200] It has been shown to be transferable by mating experiments with *S. pyogenes* as the recipient.[208] ϕm46.1 is widespread among *S. pyogenes*, but while ϕm46.1 has not been detected in any other streptococcal species outside of *S. pyogenes*,[199] it has been shown to be transferable to *S. agalactiae*, *S. suis*, and *S. gordonii* with negligible fitness cost, and in the case of *S. suis*, ϕm46.1 appeared to grant the species a noticeable fitness advantage.[199] On the other hand, no transfer was demonstrated when *S. pneumoniae*, *Streptococcus oralis*, *S. salivarius*, and *E. faecalis* were the recipients.[199,208]

2.4.1.2.4 *mef*(E)

mef(E) was first reported in *S. pneumoniae* shortly after the first description of *mef*(A).[182] *mef*(E) exhibits >90% homology to the *mef*(A) detected in Tn*1207.1*.[115] As described previously, the distinction of *mef*(E) from *mef*(A), in spite of the high degree of homology, has been justified by many researchers in part by the distinct mobile genetic elements that encode these genes. *mef*(E) has since been reported as being widespread among *S. pneumoniae* (with some isolates being detected with *mef*(E) and *erm*(B)) and has been detected in a variety of other species,[193] including *S. pyogenes* and *S. aureus*.[115,192,209-211]

A number of studies have investigated the prevalence of *mef*(E) among particular groups of macrolide resistant isolates. In one study, from a collection of 99 erythromycin resistant *S. pneumoniae* isolates from healthy Polish children aged 3-5 years, eight carried *mef*(E) (all

alongside *erm*(B)).[212] From a collection of 69 macrolide resistant *S. pneumoniae* isolates obtained via the PROTEKT study over the period of 1999-2000, all of which had previously been identified as both *erm(B)* and *mef* positive, 18 were found to carry *mef*(E) rather than *mef*(A).[213] Among a collection of 70 Canadian paediatric multi-resistant *S. pneumoniae* isolates collected over the period of 1998-2004, 66.2% carried *mef*(E) and 8.5% carried *mef*(E) and *erm*(B).[214] Jeric et al. (2007) detected *mef*(E) in nine MRSA isolates out of a small collection of 11 nosocomial isolates.[209] Silva-Costa et al. detected *mef*(E) in a single isolate out of a collection of 161 *S. pyogenes* isolates exhibiting the M-phenotype collected over the period of 2007-2013; none had been detected in a collection of 156 isolates collected over the period of 2004-2006.[44,80] Mazzariol et al. (2007) found that 30.8% of a collection of 26 clinical isolates of *S. pneumoniae* exhibiting the M phenotype carried *mef*(E).[103]

Induction of the *mef*(E)/*msr*(D) system is controlled at the level of transcription. It has been suggested that induction of *mef*(E) is controlled by transcriptional attenuation;[98,215] a putative leader peptide encoded upstream of *mef*(E) shares similarities to the leader peptide of the transcriptionally-attenuated *erm*(K).[98] *mef*(E)/*msr*(D) can be induced by the 16-membered macrolides tilmicosin and rosamicin,[215] in addition to 14- and 15-membered macrolides. Given that none of telithromycin, tilmicosin or rosamicin contain a C3 cladinose, it has been suggested that cladinose is not necessary for *mef*(E)/*msr*(D) to be induced.

It is worth noting that *mef*(E) can also be induced by the macrophage-borne antimicrobial peptide LL-37, possibly via a different pathway than macrolide-mediated induction, suggesting that induction may occur at the site of infection.[98] This may imply that the degree of *mef*(E)-mediated resistance in bacterial isolates carrying this particular gene may be much higher in the clinical setting than would be suggested by *in vitro* MIC testing.[216]

2.4.1.2.5 Mobile genetic elements carrying *mef*(E)

While *mef*(E) share a high degree of homology with *mef*(A), the elements one which it has been described differ significantly. As described by Gay and Stephens (2001), the genetic element carrying *mef*(E) in *S. pneumoniae* is mega (macrolide efflux genetic assembly).[184] It contains 5 ORFs that are closely related to the last 5 ORFs of the *mef*(A)-carrying Tn*1207.1*. The first of these 5 ORFs is *mef*(E) and the second ORF is *msr*(D) (previously referred to as *mel*). There are a number of insertion sites that have been described within the genome of *S. pneumoniae*,[184] with each site possibly being associated with different variants of mega.[115]

In other instances of *mef*(E)-carrying *S. pneumoniae*, the *mef*(E)-carrying element was found to be a composite element where mega had been inserted in another transposon. Examples of these composite transposons include Tn*2009* and Tn*2010*, where mega is inserted within *orf6* of the *tet*(M)-carrying elements Tn*916*[217] and Tn*6002*,[137,139] respectively, and oriented in the opposite direction of *tet*(M).[115] These elements provide a vehicle for the simultaneous transfer of *tet*(M), *mef*(E) and (in the case of Tn*2010*) *erm*(B). Indeed, streptococcal isolates carrying both *mef*(E) and *erm*(B) together have been detected. In both Tn*2009* and Tn*2010*, mega was similar in composition to a mega variant reported in *S. salivarius* that contained an additional ORF (*orf6*) between *orf4* and *orf5*.[218] In vitro attempts at conjugated either element have been unsuccessful,[137] although Tn*2009* has been shown to be transformable.[217]

2.4.1.2.6 Other *mef* genes

Mosaic *mef*(A)/*mef*(E) variants of *mef*, in which the 5' end is identical to *mef*(A) and the 3' end is identical to *mef*(E), have been detected in *S. pyogenes*, and were shown to be consistently associated with *tet*(O).[191] The element carrying this mosaic *mef*(A/E) was found to be a variant of ϕm46.1.

The *mef*(I) gene, detected in *S. pneumoniae*, *S. pyogenes* and group C streptococci, exhibits >90% homology to both *mef*(A) and *mef*(E).[186,219] *mef*(I) is carried on a composite element named 5216IQ complex. The complex consists of an element (IQ) inserted into a defective variant of Tn*916*, which is in turn inserted into a defective variant of Tn*5252*. The resulting element (30505 bp) consists of two halves. The left half (15316 bp) is formed from segments of Tn*5252* and Tn*916*, in which the *tet*(M) is silent. The right half (15115 bp) contains *mef*(I) adjacent to its own *msr*(D) variant, along with the chloramphenicol resistance gene *catQ*, marking the first time this gene was detected and linked with *mef* in *S. pneumoniae*.[220] Induction of *mef*(I) has not been demonstrated.[98]

Finally, *mef*(O), *mef*(B), *mef*(C) and *mef*(G) are additional *mef* gene variants that have recently been described in streptococci, all associated with their own *msr*(D) variants. *mef*(B) was initially described in *S. agalactiae*;[219] the Roberts database does not list *Streptococcus* as an associated genera with *erm*(B) but does list *Escherichia*.[95] *mef*(G) has been detected in *S. agalactiae* and *Streptococcus dysgalactiae*.[219,221] The elements that carry these *mef* genes have not been characterized to the same extent as *mef*(I).

2.4.1.2.7 Other acquired efflux genes

Aside from the *mef* gene group, numerous other AMRGs coding for efflux mechanisms with action against MLS_B antibiotics have been described, although many of them are less commonly encountered among common respiratory pathogens than *mef*. Many of these mechanisms are not major facilitators like *mef*, but are instead ATP-binding transporters.[95] *msr*(A) was among the first of these described. Originally reported in *S. epidermidis*, studies have commonly encountered this gene in macrolide resistant isolates of *S. epidermidis*,[222] and it is also frequently reported in clinical isolates of *S. aureus*.[223] In addition, *msr*(A) has been detected in *Streptococcus* spp., *Corynebacterium* spp. and *Enterococcus* spp. (Roberts 2008, Chancey 2012).[1,98] *msr*(A) typically has action against erythromycin and streptogramin B,

but not against 16-membered macrolides or lincosamides.[95,98,224] Due to similarities between the leader peptides of *msr*(A) and *mef*(E), it is believed that induction of *msr*(A) is controlled at transcription.[98] A number of plasmids have been identified as carriers of *msr*(A), including the *msr*(A)-, *erm*(Y)- and *mph*(C)-bearing pMS97 detected in *S. aureus*[160,161] and pUL5054 detected in *S. epidermidis*.[95,183] Other *msr* variants have also been described, including *msr*(C), *msr*(D) (found in a linkage with *mef*) and *msr*(E).

Another macrolide efflux gene, *car*(A), results in efflux against carbomycin, an uncommonly used 16-membered macrolide with a similar range of activity to that of erythromycin. Other ATP-binding transporter gene groups include *lsa*, *vga* and *ole*, though these tend to be more potent against particular lincosamides and streptogramin antibiotics rather than erythromycin and other macrolides. *lmr*(A), like *mef*, is a major facilitator rather than an ATP-binding transporter, but is more potent against lincomycin than erythromycin.[1,95,97]

2.4.1.3 Macrolide inactivation

A third class of genes has been described that also have inhibitory activity against macrolides and other antibiotics classified under the MLS_B group. These genes code for enzymes that inhibit the drug by acting on them directly and deactivating them rather than by altering the macrolide binding site or by efflux action (see Table 2.7). The *mph* genes encode a phosphorylase that inactivates macrolides through the introduction of a phosphate on the 2'-hydroxyl group of the amino sugar.[225] At least seven *mph* genes have been described. They have typically been reported among in Gram negative bacteria,[95] but they have also been reported in Gram positives and *mph*(C) in particular has been reported among *S. epidermidis* of human and animal origin.[222,226,227] A recent study by Juda et al. (2016) detected *mph*(C) among erythromycin-resistant *S. epidermidis* respiratory isolates from lung cancer patients. Most instances of the genes were in combination with *erm* genes and/or *msr*(A); 2 isolates

Table 2.7: List of currently identified* MLS_B inactivating enzyme-encoding genes and the genera in which they have been detected.[95]

Gene	Enzyme Type	Resistance Profile	Genera
ere(A)	Esterase	Erythromycin	*Achromobacter*, *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Laribacter*, *Pantoeae*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Staphylococcus*, *Stenotrahomonas*, *Vibrio*
ere(B)	Esterase	Erythromycin	*Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Staphylococcus*
vgb(A)	Lyase	Streptogramin B	*Enterococcus*, *Staphylococcus*
vgb(B)	Lyase	Streptogramin B	*Staphylococcus*
lnu(A)	Transferase	Lincomycin	*Staphylococcus*, *Clostridium*, *Lactobacillus*, *Pediococcus*
lnu(B)	Transferase	Lincomycin	*Enterococcus*, *Erysipelothrix*, *Staphylococcus*, *Clostridium*, *Streptococcus*
lnu(C	Transferase	Lincomycin	*Streptococcus*, *Haemophilus*
lnu(D)	Transferase	Lincomycin	*Streptococcus*
lnu(E)	Transferase	Lincomycin	*Streptococcus*
lnu(F)	Transferase	Lincomycin	*Escherichia*, *Salmonella*
lnu(G)	Transferase	Lincomycin	*Enterococcus*
lnu(P)	Transferase	Lincomycin	*Clostridium*
vat(A)	Transferase	Streptogramin A	*Staphylococcus*
vat(B)	Transferase	Streptogramin A	*Enterococcus*, *Staphylococcus*
vat(C)	Transferase	Streptogramin A	*Staphylococcus*
vat(D)	Transferase	Streptogramin A	*Enterococcus*
vat(E)	Transferase	Streptogramin A	*Enterococcus*, *Lactobacillus*
mph(A)	Phosphorylase	Macrolides	*Aeromonas*, *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pantoeae*, *Pseudomonas*, *Proteus*, *Serratia*, *Shigella*, *Stenotrophomonas*
mph(B)	Phosphorylase	Macrolides	*Escherichia*, *Enterobacter*, *Pseudomonas*, *Proteus*
mph(C)	Phosphorylase	Macrolides	*Staphylococcus*, *Stenotrophomonas*
mph(D)	Phosphorylase	Macrolides	*Escherichia*, *Klebsiella*, *Pantoeae*, *Proteus*, *Pseudomonas*, *Stenotrophomonas*
mph(E)	Phosphorylase	Macrolides	*Acinetobacter*, *Citrobacter*, *Escherichia*, *Klebsiella*, *Pasteurella*, *Serratia*
mph(F)	Phosphorylase	Macrolides	*Pseudomonas*
mph(G)	Phosphorylase	Macrolides	*Photobacterium*, *Vibrio*

*Current as of December 16th 2016

with *mph*(C) alone presented with an MS_B phenotype.[222] The *ere* genes, of which *ere*(A) and *ere*(B) are described and which have also been historically associated with Gram negative organisms, code for esterase enzymes that hydrolyze the lactone ring of the macrocyclic nucleus.[225] *ere*(A) has been reported among a slightly wider variety of species than *ere*(B).[95] Ere-like activity has been reported in clinical isolates of *S. aureus* for some time[228] and both have been detected in *S. aureus* and coagulase-negative staphylococci from animal sources.[229] *ere* primarily produces high-level resistance to erythromycin. The *mph* and *ere* genes have typically been plasmid-borne,[95] although *mph*(E) has also been reported chromosomally in *P. multocida* sourced from animals.[230]

Other genes encoding deactivating genes have been described, but these have not been widely reported to have action against macrolides. The lyase-encoding *vgb*(A) and *vgb*(B) are associated with streptogramin B resistance while the transferase-encoding *lnu* and *vat* genes are associated with lincomycin and streptogramin A resistance, respectively. These genes have also been detected on plasmids.[95]

2.4.2 Ribosomal binding site alterations associated with macrolide resistance

In addition to AMRGs, a number of chromosomal mutations have also been associated with the development of macrolide resistance. These mutations typically occur in regions associated with structural components of the bacterial ribosome, and the resulting changes to the ribosomal binding site of the macrolides ultimately reduce the binding affinity of the macrolide to the ribosome. The most commonly described chromosomal alterations occur in genes coding for the L4 and L22 ribosomal protein genes, both of which code r-proteins that are components of the 50S subunit of the ribosome,[231] and from mutations in the 23S rRNA segment of the 50S segment of the bacterial ribosome,[1] the most common target for

antibiotics which act by inhibiting protein synthesis. This is somewhat reminiscent of the effect of the *erm* gene group, although the resistance phenotype produced by these mutations will not necessarily be the same as that of *erm*. These mutations have been shown to develop under antibiotic pressure[232] and can be passed on to daughter cells, but generally cannot be passed on horizontally to different genera.[1]

2.4.2.1 L4 and L22

The presence of ribosomal protein alterations have been reported in macrolide resistant isolates for some time. The proteins that have been the most consistently researched have been the L4 and L22 proteins, which lie within the peptide exit tunnel of the 50S subunit. Macrolides bind in a narrow part of this tunnel between the peptidyl transferase centre and a constriction in the tunnel near L4 and L22, with some macrolides and macrolide derivatives making contact with these proteins.[4] As a result these proteins are believed to play a significant role in the binding of macrolides to the ribosomal binding site.[233]

Alterations in these proteins can hinder the ability of macrolides to bind to the ribosome. Alterations in highly-conserved regions of these proteins as a result of mutations in the genes coding for them (including single base substitutions, insertions and deletions) have long been reported to be associated with macrolide resistance in a number of species including *E. coli*.[96] Chittum and Champney (1994) reported that an A to G substitution at the first position of codon 63 in the L4-encoding *rplD* resulted in a lysine to glutamic acid change in L4 in an erythromycin-resistant *E. coli* mutant, whereas a nine base pair deletion resulting in a three codon loss in the L22-encoding *rplV* resulted in an altered L22 in another erythromycin-resistant *E. coli* mutant.[234] Other Gram negative species in which L4 and/or L22 alterations have been reported and associated with macrolide resistance include *Campylobacter* spp. and *Rickettsia* spp..[1]

Among Gram positive organisms, L4 and/or L22 alterations have been reported in *E. faecalis*, *B. subtilis*, *S. aureus* and *S. pyogenes*.[1] L4 and L22 alterations generated in pneumococci *in vitro* have been shown to be associated with resistance. Tait-Kamradt et al. (2000) were able to generate two *S. pneumoniae* mutants bearing alterations in L4. One mutant contained a C69D alteration, while the other mutant contained an INS 67SQ. Both mutants saw an approximate 4-fold increase in MICs of 14-, 15- and 16-membered macrolides and streptogramin B compared to a susceptible isolate, but lincosamide and telithromycin MIC saw more modest increases.[232] In addition, clinical strains isolated from Europe were found to carry a three amino acid substitution (69GTG71 → TPS) in L4 and were associated with an MS_B phenotype (resistance to macrolides and streptogramin B only). Another strain from Canada with a similar phenotype carried an insertion of six amino acids (REKGTG) after position 71. The macrolide MICs for this Canadian isolate were not as high as the European isolates and the alteration appeared to have an impact on growth time, although the telithromycin MIC was higher.[235] L22 alterations such as G95D, P99Q, A93E, P91S and G83E have been associated with resistance to streptogramin B and low levels of clindamycin and macrolides in *S. pneumoniae*.[236]

2.4.2.2 23S

Direct chromosomal alterations of the binding site of macrolides are also known to reduce the susceptibility of bacteria to macrolides. 23S rRNA alterations have been reported in a variety of Gram positive and Gram negative species, including but not limited to *S. aureus*, *S. pyogenes*, *S. pneumoniae*, *E. coli*, *Neisseria gonorrhoea*e and *Campylobacter* spp..[1]

Among the most widely reported and investigated 23S rRNA alterations are those of A2058 in domain V (using *E.coli* numbering), a nucleotide residue that serves as a common binding site for macrolides. In *S. pneumoniae*, an A2058G alteration has been shown to be associated

with the MLS_B phenotype in resistant isolates generated *in vitro*,[232] and a similar effect was observed in *Propionibacterium* spp..[237] A2058G has also been reported in a variety of other species,[238] including *E. coli* (associated with resistance to erythromycin and lincosamides),[231] *Mycobacterium* spp. (associated with resistance to clarithromycin and occasionally azithromycin)[239,240] and *Helicobacter pylori* (associated with the MLS_B phenotype).[241,242] Other alterations of the wild-type A2058, including A2058C and A2058U, have also been associated with macrolide resistance in these species.[238] Furthermore, alterations at adjacent positions such as G2057 and A2059 have also been observed and associated with macrolide resistance;[238] in *S. pneumoniae*, an A2059G alteration was associated with an azithromycin MIC of >200 µg/mL, but was not associated with an increase in resistance against streptogramin B like that observed with A2058G.[232] Clinical strains of *S. pneumoniae* with this alteration have also been detected and were associated with an ML phenotype (resistance to macrolides and lincosamides only).[235] G2057 alterations reportedly confer resistance to 14-membered macrolides in *E. coli* and *Propionibacterium* spp., but not to 16-membered macrolides.[238]

C2611 is another common hotspot for erythromycin resistance-inducing alterations, and such alterations confer a similar phenotype to that resulting from G2057 alterations.[238] Tait-Kamradt et al. (2000) demonstrated an association with C2611A and C2611G and macrolide resistance in isolates of *S. pneumoniae* generated via macrolide passage *in vitro*,[232] while Vannuffel et al. (1992) detected C2611U in erythromycin-resistant *E. coli*.[243] Also in *E. coli*, a U754A alteration in domain II has been shown to be associated with low level erythromycin resistance,[244] while an A752 deletion has been associated with erythromycin, azithromycin and clarithromycin MICs of >32 µg/mL in *S. pneumoniae*.[236] Alterations within domain II have also been associated with erythromycin resistance in *E. coli*, with

particular three consecutive nucleotide group deletions (including CAU1321 and AUG1232) being sufficient to confer resistance.[245]

Many species contain multiple copies of 23S. For example, *S. pneumoniae* has been shown to carry four copies of 23S.[232] Intriguingly, it has been demonstrated that it is not necessary for all of these 23S operons to carry a mutation for a macrolide resistance phenotype to develop. For example, in *Streptomyces ambofaciens*, only one of its four copies is required to carry a mutation such as A2058G to produce erythromycin resistance,[246] while it has been shown that only one copy is required to be altered in *Helicobacter* spp. and *M. pneumoniae*.[232] In the study of Tait-Kamradt et al. (2000), the C2611G mutant (in which all four copies of 23S bore the alteration) was more resistant to 14- and 15-membered macrolides, as well as streptogramin B and telithromycin, than the C2611A mutant (in which three of the four copies bore the alteration), but it was not clear if the copy number was the determinant of this difference or if it was related to the specific amino acid difference.[235]

2.5 Macrolide resistance mechanisms in NTHi

The mechanisms behind macrolide resistance in NTHi are not as well understood as in other species. The current evidence suggests that macrolide resistance in NTHi is mediated primary by chromosomal factors with comparatively few reports of AMRGs. In some studies, isolates exhibiting increased macrolide resistance lack any known chromosomal mutations or AMRGs, leaving the specific cause of the resistance in these cases unresolved. This section will examine the current evidence for both chromosomal mutations and AMRGs in macrolide-resistant NTHi.

2.5.1 Chromosomal multi-drug efflux and AcrAB

Among many species, inherent multi-drug efflux systems have been described. In NTHi, such systems include those encoded by homologs of *ydeA*, *yieO* and *norM*, although the effect of many of these systems in NTHi has been reported to be minimal.[247] The AcrA/AcrB/TolC homolog efflux system has been well described and is the primary *H. influenzae* efflux mechanism of this type.[247] It was established years ago that the *H. influenzae* genome contains a three-gene complex that is homologous to the *acrRAB* complex (consisting of homologs of *acrR* (the regulatory gene controlling expression of the complex), *acrA* and *acrB*) found in *E.coli*.[248] The AcrAB efflux complex (consisting of the outer membrane transporter TolC, the membrane fusion protein AcrA and the inner membrane transporter AcrB) is known to contribute to antibiotic efflux in *E. coli*,[249] and Sanchez et al. (1997) were able to demonstrate that expression of the homologous system contributed to the baseline macrolide MIC levels typical of wild-type strains of *H. influenzae*, and that disruptions of the *acrA* and *acrB* homologs resulted in hyper-susceptibility to erythromycin.[250] Peric et al. (2003) also reported that hyper-susceptible strains lacked any efflux mechanism such as that seen in hyper-resistant and base-line strains,[90] indicating that

this efflux mechanism may be the reason that wild-type NTHi strains have base-line MICs that are higher than is observed in base-line Gram positive species. Trepod et al. (2004) demonstrated that inactivation of the individual components of AcrAB resulted in increased susceptibility to erythromycin and other antibiotics in *H. influenzae*.[247]

Given that MICs in isolates carrying the AcrAB system and similar systems are still relatively low compared to highly-resistant organisms, there has historically been some contention as to whether this kind of macrolide efflux is clinically relevant in *H. influenzae*.[37] However, there is recent evidence that suggests that overexpression of *acrB* resulting from mutational changes can lead to high-level macrolide resistance. In particular, Seyama et al. (2016) reported that all isolates among a small collection of respiratory NTHi isolates with high-level clarithromycin resistance carried a frameshift mutation within *acrR* and exhibited increased transcription of *acrB*. No isolates were found to carry *erm*(B) or *mef*(A), and all isolates tested negative for amino acid substitutions within the L4 and L22 ribosomal proteins. In addition, transformation of the *acrR* variant into susceptible recipients resulted in the same increased *acrB* expression and clarithromycin MIC as the donor strain.[251] This outcome indicates that not only can the AcrAB system cause high-level resistance in the absence of other known mechanisms, but that *acrR* may in fact play a significant role. The authors also note that since the mutation sites within *acrR* varied among their isolates, *acrR* may act as a hotspot for such mutations.[251]

2.5.2 Ribosomal mutations

The presence of mutations in chromosomal genes coding for ribosomal structural components in macrolide-resistant NTHi isolates has been documented, and the presence of these chromosomal mutations has formed the basis of classification of NTHi isolates based on their resistance profile to macrolides. The majority of isolates that form the base-line with respect

to macrolide susceptibility for this species lack chromosomal mutations in ribosomal structural elements associated with macrolide resistance, but do exhibit inherent macrolide efflux. A small proportion of isolates lack this efflux mechanism and ribosomal alterations and are defined as hyper-susceptible isolates. Finally, occasional highly macrolide-resistant isolates typically exhibit the inherent efflux but also carry at least one ribosomal alteration.[90,252]

Peric et al. (2003) reported that 28 out of a collection of 31 *H. influenzae* isolates with high-level macrolide resistance had alterations in L4, L22 or 23S rRNA (all in domain V). Among the seven isolates with 23S rRNA alterations detected (five of which also carried L4 and/or L22 mutations), A2058G (based on *E. coli* numbering) was detected in two isolates (with one isolate carrying an additional G2160U), and the remaining isolates carried alterations within the 2160-2164 range of positions, including one isolate with a GGA2160-2162UAU alteration. The majority of these isolates with 23S rRNA alterations, including both isolates with A2058G alterations, had azithromycin and/or clarithromycin MICs of >64 µg/mL.[90] In a subsequent study including many of the same isolates as Peric et al. (2003), Bogdanovich et al. (2006) reported the presence of domain II 23S rRNA alterations among azithromycin- and clarithromycin-resistant isolates, with A654G, G884U and C894A being reported among multiple isolates. Only one isolate carried a domain II alteration (A654G) with no other alterations; this isolate had MICs of >32 µg/mL against clarithromycin and 4 µg/mL against azithromycin.[252] It is worth noting here that while NTHi is known to carry at least 6 copies of 23S rRNA, reports on the presence of 23S rRNA have typically not mentioned whether all copies carry the mutations listed here, or if only some of the copies have been altered with the other copies remaining unchanged. Therefore, it is not currently clear whether there is a link between the number of copies carrying alterations and the emergence of macrolide resistance in NTHi.

In the study of Peric et al. (2003), seven isolates solely carried an L4 protein alteration. One isolate bore an INS 63GT alteration, and the remaining six carried a single point mutation: K61Q (one isolate), T64K (one isolate), A69S (one isolate), T82I (one isolate) or G65D (two isolates). Clarithromycin MICs ranged from 32 to >64 µg/mL for these isolates, and azithromycin MICs ranged from 8 to 64 µg/mL. Eleven isolates carried a L22 protein mutation alone. Two isolates carried a G91D substitution and exhibited clarithromycin and azithromycin MICs of 16 µg/mL and 8 µg/mL, respectively. The remaining isolates bore amino acid insertions or substitutions; clarithromycin MICs ranged from 16 to >64 µg/mL, and azithromycin MICs ranged from 8 to >64 µg/mL. Another eight isolates carried alterations in multiple sites. Five of these isolates carried 23S rRNA alterations in positions 2160-2164 (based on *E. coli* numbering). A T64K alteration in L4 and a G91D alteration in L22 were paired together in five isolates; three of these five isolates also carried a C2164G alteration in 23S rRNA. Of the remaining three isolates, one carried L4 and 23S rRNA alterations, one carried L22 and 23S rRNA alterations, and one carried L4 and L22 alterations. The MICs for the eight isolates with multiple alterations ranged from 32 to >64 µg/mL against clarithromycin and from 8 to >64 µg/mL against azithromycin.[90] In a more recent study assessing the effect of macrolide and quinolones on eradication of *H. influenzae* in COPD patients and resistance emergence in persistent *H. influenzae* isolates, Pettigrew et al. (2016) reported the emergence of a G91D alteration in L22 in persistent isolates derived from patients with COPD after undergoing macrolide therapy; L22 alterations were not detected in the same patients prior to undergoing macrolide therapy. The persistent isolates that developed this L22 alteration exhibited 4-fold increases in azithromycin MIC values. L4 and 23S rRNA alterations were not detected among any isolates in the same study.[29]

Note that the presence of alterations in certain ribosomal structural elements does not appear to ensure that high-level macrolide resistance will develop in NTHi. Bogdanovich et al.

(2006) reported three isolates carrying an L4 alteration (two with K61Q and one with A69S) alongside multiple alterations of 23S rRNA in domain II (including A654G, G884U and C894A), all of which exhibited azithromycin MICs of ≤1 µg/mL and clarithromycin MICs of 8 µg/mL.[252] Interestingly, Peric et al. (2004) reported the presence of L22 R88P alterations in both high-resistant and hyper-susceptible strains of NTHi. Upon further investigation, it was found that the hyper-susceptible isolates carrying this mutation lacked the ability to pump macrolides via an efflux mechanism. This suggested that particular alterations in L22 may not be sufficient to cause high-level macrolide resistance in NTHi alone, and that it requires supplementation by an unaltered inherent efflux mechanism such as AcrAB/TolC to result in a resistance phenotype.[253] This also reaffirms the importance of inherent efflux in the development of high-level macrolide resistance in NTHi.

2.5.3 Acquired macrolide resistance genes in NTHi[†]

Historically, AMRGs have not been widely associated with macrolide resistance in NTHi, and studies reporting on macrolide resistance mechanisms in NTHi have typically been unable to detect AMRGs among macrolide-resistant isolates. In a study by Peric et al. (2004) involving 31 macrolide-resistant NTHi isolates, no isolate was found to carry *mef*(A), *erm*(A), *erm*(B), or *ere*(A).[253] In a follow-up to that study including many of the same isolates and some new isolates, Bogdanovich et al. (2006) also failed to detect the presence of *mef*(A), *erm*(A), *erm*(B), or *ere*(A).[252] More recently, Seyama et al. (2016) were not able to detect *mef*(A) or *erm*(B) among a smaller collection of 7 clarithromycin-resistant isolates, with resistance instead being attributed to increased transcription of the *acrB* gene as a result of alterations of the *acrR* regulatory gene,[251] and Pettigrew et al. did not detect *erm*(A),

[†]FOOTNOTE: The content of the literature review is based on publications up to December 16th 2016 in account of a January 6th 2017 thesis submission date. However, a very recent study – available in advance access online from December 20th 2016 – has reported the detection of an isolate of *H. influenzae* with *mef*(A).[254] This will not be discussed in the literature review or Chapters 3-7, but is briefly discussed in the general discussion of Chapter 8.

erm(B), *erm*(C), *erm*(F) or *mef*(A) in any isolates (n = 200, or 100 persistent strain pairs) included in their study (MIC increases were either attributed to L22 alterations or the mechanism was not determined).[29]

In recent years, there has been one report where AMRGs have been widely reported among NTHi isolates. In a study by Roberts et al. (2011) regarding the prevalence of particular macrolide resistance genes among CF isolates of NTHi, the authors aimed to characterise the macrolide resistance mechanisms in NTHi isolates taken from CF patients enrolled in a placebo-controlled azithromycin trial. Each isolate was tested for phenotypic macrolide resistance by E-test MIC, as well as for the presence of L4 and L22 mutations (23S rRNA was not investigated), and the *erm*(A), *erm*(B), *erm*(C), *erm*(F) and (undifferentiated) *mef*(A) genes. The authors found that 25.5% of their strains were resistant to azithromycin and erythromycin (using BSAC interpretive criteria; erythromycin MIC > 8 µg/mL; azithromycin MIC > 4 µg/mL), and 73.6% were intermediate to both macrolides (using BSAC interpretive criteria; 1 µg/mL ≤ erythromycin MIC ≤ 8 µg/mL; 0.5 µg/mL ≤ azithromycin MIC ≤ 4 µg/mL). None of these resistant strains carried L4 or L22 mutations, but 93% of all the strains included in the study (regardless of phenotypic macrolide resistance profile) carried at least one of *erm*(B) (31% prevalence), *erm*(F) (29% prevalence) and *mef*(A) (74% prevalence). The remaining isolates that were negative for these 3 genes were additionally tested for and found to carry at least one of *erm*(A) and *erm*(C), resulting in 100% of the isolates included in the study carrying at least one AMRG. The author also note that *erm*(A) and *erm*(C) prevalence may have been underestimated as only a small number of the isolates in the study were tested for these genes.[93]

Furthermore, the authors demonstrated that select donors were able to transfer these genes to *H. influenzae* Rd KW20 as well as *E. faecalis* JH2-2 via conjugation. The *H. influenzae* transconjugants demonstrated an up to a 48-fold increase in erythromycin MIC and up to a

24-fold increase in azithromycin MIC compared to the recipient, while the *E. faecalis* transconjugants demonstrated a 16-fold increase in erythromycin MIC compared to the recipient.[93] These findings suggested that these genes were associated with mobile genetic elements, and that NTHi could act as a donor and recipient of these genes. Given that these genes are detectable among respiratory bacteria and *P. aeruginosa*, the authors speculated that selective pressure via continuous antibiotic exposure associated with CF therapy might facilitate the exchange of these AMRGs *in vivo*.[93]

The ability to successfully transfer AMRGs to *H. influenzae* by conjugation was not a novel finding. Chung et al. (1999) were able to transfer *erm*(F) to *H. influenzae* RD8 (erythromycin MIC = 1 μg/mL) from both *Prevotella bivia* and *Haemophilus aphrophilus* (since re-classified as *Aggregatibacter aphrophilus*), with transconjugants exhibiting increased erythromycin MICs (64 μg/mL),[158] while Luna et al. (2000) have previously described the transfer of *mef* from *S. pneumoniae* to *H. influenzae* RD8 (phenotype changes were not reported).[255] However, this study represented the first report of *erm*(A), *erm*(C) and *erm*(F) among clinical NTHi, and the authors also state that this is the second report of *mef*(A) and *erm*(B) among NTHi isolated from CF patients.[93] Furthermore, the authors of the study go on to state that there may be some correlation between the acquisition of these genes by NTHi and the development of macrolide resistance in NTHi. This was based on the observation that 56% of the macrolide-resistant NTHi isolates in the study carried at least 2 macrolide genes. By comparison, 23% of the isolates that were defined as intermediate to macrolides carried at least 2 genes.[93] However, it is worth noting that the MIC values of many of the isolates included in the study did not appear to be highly raised compared to a typical wild-type strain despite carrying at least one AMRG. Many isolates carrying an *erm* gene were defined at "intermediate" according to the MIC results and did not demonstrate the high level resistance that would be typically be produced by these genes. Out of 7 isolates in

the study that carried all of *mef*(A), *erm*(B) and *erm*(F), only 2 had MIC values falling above the resistance breakpoint, with the remaining defined as "intermediate", and there was 1 particular isolate that carried *erm*(B) and *mef*(A) simultaneously but was susceptible to erythromycin.[93] The reason for these particular findings is not clear but suggests that there may be other factors involved that allow for these genes to grant macrolide resistance in NTHi.

2.5.4 Acquired MLS_B resistance genes in other *Haemophilus* spp.

Haemophilus parainfluenzae is closely related to *H. influenzae* and an occasional opportunistic pathogen in humans. *H. parainfluenzae*, which is differentiated from *H. influenzae* based on its lack of requirement on X factor (haemin) for growth, has been associated with a number of infections including urethritis and respiratory infections (in which macrolides may be prescribed). Notably, it is a known cause of infective endocarditis and is included in the HACEK group of fastidious Gram-negative bacteria that can cause this disease.[256] It has previously been demonstrated that *H. influenzae* and *H. parainfluenzae* are able to share mobile antibiotic resistance determinants with each other, and suggested that *H. parainfluenzae* may act as an important reservoir for antibiotic resistance genes and plasmids for *H. influenzae*.[37]

Tinguely et al. (2013) reported the presence of multi-drug resistance in 2 separate isolates of *H. parainfluenzae* (both from urethral swabs of homosexual men, isolated 5 months apart). The isolates possessed identical phenotypic and genotype characteristics. The isolates exhibited high-level resistance to erythromycin, azithromycin and clarithromycin (MIC > 256 µg/mL for all 3 drugs) and were additionally resistant to a broad range of β-lactam antibiotics (but non-resistant to meropenem), ciprofloxacin and levofloxacin, tetracycline, and chloramphenicol. The isolates were susceptible to cotrimoxazole and rifampin. Analysis of

the isolates revealed that both carried *mef*(A) and an L4 mutation (A69S). The authors did not detect any known macrolide resistance-associated alterations in L22 or 23S, and *erm*(A), *erm*(B), *erm*(C), *erm*(D) and *erm*(F) were not found.[257] A subsequent report revealed that *mef*(A) was carried on the mega element.[258]

The authors attribute the high-level resistance to macrolide in the isolates to the presence of *mef*(A) and the A69S alteration of L4.[257] However, it is not certain how much each mechanism was contributing to the reported phenotype. The *mef* genes typically produce a moderate increase in erythromycin MIC and are not normally associated with high-level resistance across all of erythromycin, azithromycin and clarithromycin. Meanwhile, the role of macrolide resistance-associated chromosomal mutations have not been widely investigated in *H. parainfluenzae*, although in *H. influenzae*, the A69S was previous reported in an isolate with azithromycin and clarithromycin MICs of 32 µg/mL.[90] On the other hand, A69S has also been reported (in parallel with multiple mutations in domain II of 23S) in an *H. influenzae* isolate with azithromycin and clarithromycin MICs of 0.5 µg/mL and 8 µg/mL, respectively.[252] The high MICs observed here may be attributable to a combined effect between the L4 mutation and *mef*(A). The combined effect of chromosomal mutations and AMRGs on phenotypic macrolide resistance has not been widely studied, although the previously discussed report of the inability of L22 to increase macrolide resistance in the absence of an underlying efflux (such as acrAB)[253] does provide a precedent for the possibility of particular mechanisms having a greater effect on macrolide resistance when in the presence of another mechanism to supplement it.

Haemophilus parasuis is the causative agent of Glässer's disease in swine. Chen et al. (2010) have reported the presence of the transferase-encoding lincosamide resistance gene *lnu*(C) in an isolate of *H. parasuis* exhibiting high MICs of lincomycin and clindamycin, but a low MIC of erythromycin. *lnu*(C) was carried on a 6320bp-long plasmid referred to as pHN61;

transformation of this plasmid into susceptible isolates resulted in an increase in MICs of lincomycin (2 to 32 µg/mL) and clindamycin (2 to 16 µg/mL).[259] pHN61 appeared to share little homology with plasmids previously reported in other *H. parasuis* at the time, but shared similarities with the multi-resistance plasmid pHB0503 found in *Actinobacillus pleuropneumoniae*,[260] a respiratory pathogen also found in swine. In addition, Yang et al. (2013) reported the presence of *erm*(T) in an isolate of *H. parasuis* which exhibited high-level resistance against erythromycin and lincomycin. This was the first time *erm*(T) had been reported in a Gram-negative organism. The gene was carried on a 7577bp-long plasmid that additionally carried the bla_{ROB-1} gene coding for β-lactam resistance. The regions of this plasmid, referred to as pFS39, that surrounded *erm*(T) and bla_{ROB-1} were similar to those of pHN61. *erm*(T) was constitutively expressed in this instance due to a deletion of the leader peptide and IR-1. Transformation of this plasmid into susceptible *H. parasuis* resulted in a 64-fold increase in erythromycin MIC and a 16-fold increase in lincomycin MIC, along with MIC increases for penicillin, amoxicillin and cefaclor.[261]

2.5.5 Acquired MLS_B resistance genes in members of the family *Pasteurellaceae*

Haemophilus spp. belong to the family *Pasteurellaceae* which includes a variety of closely related Gram negative rods that typically live as commensals in a number of mammal and bird species and generally survive poorly outside of this niche. Other genera of this family include *Pasteurella*, *Mannheimia* and *Actinobacillus*. Antibiotic resistance determinants, including those associated with macrolide resistance, have been documented in members of this family.[95]

P. multocida is an animal commensal known for its multi-host associations.[262] It can act as a pathogen in a number of species; in humans, it is associated with wound infections from dog

and cat bites, although *P. multocida*-mediated bacteraemia and meningitis can also develop. In recent years, multiresistant isolates of *P. multocida* derived from bovine respiratory infections have emerged, and many of these isolates also exhibit resistance to macrolides. Michael et al. (2012) have identified the presence of an integrative and conjugative element (ICE) which encodes 12 antibiotic resistance genes, designated ICE*Pmu1*, in the chromosome of a representative *P. multocida* isolate.[263] Many of these genes are not indigenous to *Pasteurellaceae* but had been previously reported among member of the family *Enterobacteriaceae*.[264] Among those genes found on ICE*Pmu1* were the macrolide resistance genes *erm*(42), *msr*(E) and *mph*(E). In a subsequent study, the same group were able to demonstrate conjugation of ICE*Pmu1* from the original representative isolate to another *P. multocida*, as well as to isolates of *Mannheimia haemolytica* and *E. coli*, with transfer of all resistance genes.[264] It is currently unclear if ICE*Pmu1* can also be conjugated to *H. influenzae*, however.

These three AMRGs are detectable among field isolates of *P. multocida* and *M. haemolytica* and can occur in different combinations that ultimately impact on the resistance phenotype. Desmolaize et al. (2011) reported three distinct phenotypes. The first phenotype is the classic MLS_B phenotype, in which high-level lincosamide resistance and low-to-moderate macrolide resistance is observed. This is attributable to monomethylation of nucleotide A2058 of 23S rRNA. Isolates with this phenotype carry *erm*(42) alone. The second phenotype involves resistance against 14- and 15-membered macrolides (including tulathromycin, used to treat bovine respiratory disease (BRD)), but comparatively less resistance against lincosamides and 16-membered macrolides (including tilmicosin, also used for BRD). This phenotype is attributable to the presence of *msr*(E)-mediated efflux and *mph*(E)-mediated phosphorylation of the antibiotics. The third phenotype involves high-level resistance across the entire MLS_B group; isolates with this phenotype carry all three genes.[265]

Members of the *Pasteurellaceae* are capable of exchanging genetic material via horizontal transfer. Of particular note is that there is precedent for the possibility of *Pasteurella* spp. and *Haemophilus* spp. sharing resistance determinants. The $bla_{ROB\text{-}1}$-carrying plasmid pB1000 was first described in *H. parasuis* in swine,[266] and it (and its derivatives) was subsequently reported in animal-derived *P. multocida* isolates and in human clinical *H. influenzae* (including NTHi) isolates in Spain.[262,267] Tristram et al. (2010) subsequently demonstrated that $bla_{ROB\text{-}1}$ is also carried on pB1000 in *H. influenzae* isolates outside of Spain.[268] This not only suggests that pB1000 plays a significant role in the spread of this resistance gene (possibly via multi-host spread of *P. multocida*), but that the spread of resistance determinants between *Pasteurella* spp. and *Haemophilus* spp. on conjugative genetic elements may be a distinct possibility. Given the previous detection of AMRG-carrying conjugative elements such as ICE*Pmu1* in *P. multocida*, there is a real possibility of NTHi also acquiring genes such as *erm*(42), *msr*(E) and *mph*(E) and subsequently exhibiting macrolide resistance.

2.6 Chapter summary

Macrolides have been used for decades for their antibiotic and anti-inflammatory properties, and they have a wide variety of applications in the clinical setting, including in the management of chronic respiratory conditions. As a result of our increasing reliance on these antibiotics, however, the proportion of macrolide-resistant species has been increasing. The prevalence of resistance reported among surveillance studies varies depending on the species, country of origin and the specific methodology used to define resistance, but regardless the prevalence continues to rise at an alarming rate in certain contexts and remains a significant threat.

A vast amount of data has been presented regarding the mechanisms involved in macrolide resistance, with resistance being attributed to both chromosomal alterations, in particular ribosomal structural elements, and the acquisition of macrolide resistance genes. Over 100 AMRGs have been identified and code for a number of different mechanisms, including rRNA methyltransferases that alter the ribosomal binding site of macrolide (e.g. *erm*), other enzymes that deactivate the drug directly, and macrolide efflux systems (e.g. *mef*). Many of the currently identified AMRGs are known to be carried on mobile genetic elements which has allowed for the horizontal transfer of these genes and their associated phenotype among isolates of different species. This has contributed to the increasing prevalence of resistance among clinical isolates.

While the mechanisms have been thoroughly studied in many species, the mechanisms of macrolide resistance in NTHi have only recently been investigated in depth. There is sound evidence for the role of chromosomal mutations in macrolide-resistant NTHi isolates. The role of AMRGs in NTHi is a contentious issue, but while these genes have not been widely

reported in this particular species, more recent evidence is suggesting that the occurrence of these genes in NTHi may be an emerging threat.

There are a number of gaps in the literature regarding our basic understanding of the mechanisms behind macrolide resistance in NTHi, such that the specific aims of research studies presented in this thesis are to:

- Investigate the prevalence of select acquired macrolide resistance genes commonly encountered among respiratory species (and previously reported in NTHi) (Chapter 4) and among animal pathogens closely related to NTHi (Chapter 7).
- Investigate the presence of a wider range of AMRGs among NTHi by analysing previously-collated whole genome sequences (WGS) of NTHi (Chapter 5).
- Investigate the phenotypic effect and expression of select AMRGs commonly encountered among respiratory species (Chapter 6).
- Investigate the potential for NTHi to acquire AMRGs from closely-related animal pathogens such as *P. multocida* by assessing the conjugative potential of ICE*Pmu1* between *P. multocida* and NTHi, and determine the stability of ICE*Pmu1* in NTHi (Chapter 7).

Chapter 3: General Methodologies and Materials

3.1 Introduction

While chapter-specific methodologies are provided subsequently in this thesis, the following section describes general methodologies used throughout this work, including media preparation, antibiotic resistance testing methods, and DNA and RNA extraction. Where necessary, methodology descriptions in subsequent chapters will refer the reader back to this chapter.

3.2 Culture media and incubation conditions

A number of different culture media were used throughout the work described in this thesis. These are outlined below.

3.2.1 Blood agar

Columbia agar (Oxoid) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. The agar was cooled to 55°C and supplemented with defibrinated horse blood to a final concentration of 5% (v/v) before pouring. If the addition of antibiotics was necessary, these were added alongside the blood.

3.2.2 Chocolate agar

Columbia agar (Oxoid) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. The agar was cooled to 80°C and supplemented with defibrinated horse blood to a final concentration of 8% (v/v). The agar was then cooled to 55°C and supplemented with Vitox (Oxoid) at 1% (v/v) before pouring. If the addition of antibiotics was necessary, these were added alongside the Vitox. Chocolate agar was primarily used for the culture of NTHi and *P. multocida*. These isolates were grown at 37°C overnight in a CO_2-rich environment.

3.2.3 Heart infusion broth

Heart infusion broth (BHI) (Oxoid) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. For the growth of NTHi, Vitox (Oxoid) at 2% (v/v) and Haemophilus Test Medium (HTM) supplement (Oxoid) to a final concentration of 15 µg/mL NAD and haematin were added to the BHI prior to inoculation, to give supplemented BHI (sBHI). If the addition of antibiotics was necessary, these were added alongside the Vitox and HTM supplement.

For all study isolates, culture in sBHI was performed at 37°C overnight on a shaker (150 rpm).

3.2.4 LB agar and broth

LB broth with agar (Lennox) (Sigma) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. The agar was cooled to 55°C before pouring. If the addition of antibiotics was necessary, these were added immediately before pouring.

LB broth base (Lennox L Broth base) (Invitrogen by Life Technologies) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions.

3.2.5 Mueller-Hinton agar and broth

Mueller-Hinton agar (Oxoid) was used for EUCAST susceptibility testing. Mueller-Hinton agar (Oxoid) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. The agar was cooled to 55°C before pouring. For the growth of NTHi, defibrinated horse blood to a final concentration of 5% (v/v) and HTM supplement (Oxoid) to a final concentration of 20 µg/mL NAD were added was added to the agar immediately before pouring (MH-F agar).

Mueller-Hinton broth (Oxoid) was reconstituted in distilled water and autoclaved in accordance with the manufacturer's instructions. HTM supplement (Oxoid) was added to the broth prior to inoculation. The broth was additionally supplemented with 5% yeast extract (Oxoid).

3.2.6 Antibiotic preparation for media

Antibiotic supplementation was required for a number of experiments performed throughout this thesis, the majority of which were prepared fresh. Table 3.1 lists the antibiotics used in

Table 3.1: Antibiotics used in the various media of this work, with corresponding solvents.

Antibiotic	**Solvent**
Tetracycline	Water
Rifampicin	Methanol
Nalidixic Acid	NaOH, 0.1M
Cefotaxime	Water
Kanamycin	Water
Erythromycin	Water
Azithromycin	Water

the various media used throughout this thesis and the solvents used to prepare each antibiotic.[269] Water was used as a diluent for all of these antibiotics as required. All antibiotic stock powders were manufactured by Sigma.

3.3 Antibiotic susceptibility testing

A number of different methodologies were used to test for antibiotic susceptibility throughout this work; the specific method used for each experiment will be indicated in the subsequent chapters of this thesis.

3.3.1 Etest

Etests on *H. influenzae* strains were performed on MH-F agar, incubated at 37°C in 5% CO_2 for 24 hours using *H. influenzae* ATCC 49247 as a control. EUCAST breakpoints were used for interpretation where relevant.[69]

3.3.2 Broth microdilution

In Chapters 6 and 7, MICs for study isolates were determined by broth microdilution in accordance with CLSI recommendations and methodology.[270]

3.4 PCR and DNA preparation

PCR reactions were performed using the C1000 Touch Thermal Cycler (Bio-Rad). Real-time PCR was performed with the CFX96 Real-Time System (Bio-Rad) using SYBR Green (Bio-Rad) unless otherwise stated. Primer sequences and annealing temperatures are described in subsequent chapters of this work.

For all analyses, genomic DNA was extracted using the Isolate II Genomic DNA Kit (Bioline) in accordance with the manufacturer's instructions.

3.5 Sanger sequencing and primer design

Sanger Sequencing was performed on PCR-generated DNA products; agarose gel electrophoresis was used to confirm the presence of amplicons of the appropriate size. The DNA was purified using the QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. DNA samples were quantified using agarose gel electrophoresis and were subsequently submitted to the Australian Genome Research Facility Ltd (AGRF) (Queensland, Australia) for sequencing. Samples were sent off in a 1.5 mL tube with 9.6pmol of the appropriate primer (2 tubes were sent off per sample, with one tube containing the forward primer and the other tube containing the reverse primer) and water to make the total volume up to 12 mL. The amount of DNA product sent off was determined in accordance with the recommendations of the AGRF (see Table 3.2 for details).

3.5.1 Sequence analysis

The AGRF provides 4 separate files for each submitted tube:

- sample.ab1: The raw chromatogram trace file
- sample.seq: A text file of the sequence, as generated by the sequencing instruments
- sample.fa: A trimmed FASTA formatted text file. Reads are trimmed on the basis of the quality values assigned to the basecalls.
- sample.bn: A BLAST file (GenBank) of the trimmed FASTA file.

When files were received, the ab1 file was first inspected for quality using the Chromas (McCarthy 1996-1998) chromatogram viewer. If the trace file was deemed satisfactory, the fa file for each sample was accessed with CLC Main Workbench 7. For each sample submitted, the fa file for both submitted tubes (containing the forward and reverse primers) was opened with CLC Main Workbench 7 and Contig files were assembled to generate the complete sequence of the PCR product. Any conflicts between the 2 trimmed sequences were resolved based on the ab1 trace files. The identity of the assembled sequence was

Table 3.2: Recommended DNA Quantities for submission to the AGRF for sequencing.

Template	Recommended Quantity of DNA
PCR Product 100-200 bp	3-8 ng
PCR Product 200-400 bp	6-12 ng
PCR Product 400-600 bp	12-18 ng
PCR Product 600-800 bp	18-30 ng
PCR Product >800 bp	30-75 ng

confirmed using Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information (NCBI).

If the aim of the sequencing was to detect sequence differences between the submitted samples and a reference sequence, a fasta file of the reference sequence was obtained from GenBank and opened with CLC Main Workbench 7. Sample sequences were aligned with the reference sequence to detect the presence of any sequence variations.

3.5.2 Primer design

Primers were designed with CLC Main Workbench 7 using an appropriate nucleotide sequence as the template. Primer quality and specificity was analysed using Primer BLAST through the NCBI.

3.6 RNA extraction and conversion to cDNA

3.6.1 RNA extraction

Cells were grown in sBHI, with appropriate antibiotics where necessary, until the bacteria had reached a point of mid-log phase growth (Abs_{650nm} = 0.200-0.800). After growth and prior to RNA isolation, RNA was stabilised by treating the cells with RNAprotect® Bacteria Reagent (Qiagen) in accordance with the manufacturer's instructions. After stabilisation, RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Extractions were performed on the QIACUBE automated platform and included a DNase treatment step for the removal of potential contaminant genomic DNA in the samples. Extracted RNA samples were stored at -80°C.

3.6.2 RNA quality analysis and quantification

RNA quality was analysed using the Experion™ Automated Electrophoresis Station (Bio-Rad) in accordance with the protocol outlined in the Experion™ RNA StdSens Analysis Kit (Bio-Rad). RNA concentrations were determined using the Qubit® 2.0 Fluorometer (Invitrogen by Life Sciences).

3.6.3 Reverse transcription to cDNA

RNA samples were reverse transcribed to cDNA using the iScript (Bioline) in accordance with the manufacturer's instructions. Prior to PCR analysis for measurement of expression, all cDNA samples were diluted 1/5 in RNase and DNase free molecular water. Samples were stored at -20°C.

3.7 Conjugation

Donor and recipient strains were grown overnight without shaking in 5.5 mL and 21.5 mL of BHI or sBHI (for *H. influenzae*). After incubation, the donor and recipient cells were mixed in a donor:recipient of 1:5 (4 mL donor with 20 mL recipient). This mixture was centrifuged at 3800 rpm for 10 minutes and the supernatant was discarded. The pellet was resuspended in 30 mL of pre-warmed (37°C) BHI before being dispensed onto the centre of a dry pre-warmed (37°C) suitable agar plate. The pellet was allowed to dry on the plate for 30 minutes; plates were subsequently incubated at 37°C for 6 hours in a suitable atmosphere.

After incubation, the bacterial growth was harvested and suspended in 1 mL of cold saline. From this suspension, a dilution series (10^{-1} to 10^{-8}) was prepared. 100 µL of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread onto suitable non-selective agar plates. 100 µL of the 10^{0}, 10^{-1} and 10^{-2} dilutions were spread onto agar plates supplemented with antibiotics suitable for the selection of transconjugants. In instances where it was necessary to calculate the conjugative transfer efficiency, 100 µL of the 10^{-3}, 10^{-4} and 10^{-5} dilutions were spread onto agar plates supplemented with an antibiotic suitable for the selection of the donor strain, while 100 µL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread onto agar plates supplemented with an antibiotic suitable for the selection of the recipient strain. Transfer efficiency was calculated as the number of transconjugant cells per recipient cell.

The specific antibiotics used throughout this work and the specific concentrations at which they were used are listed in Table 3.3.

Table 3.3: Antibiotics used for conjugation assays with corresponding concentrations.

Antibiotic	**Concentration**
Tetracycline	15 µg/mL
Rifampicin	50 µg/mL
Nalidixic Acid	8 µg/mL
Cefotaxime	1 µg/mL
Kanamycin	30 µg/mL
Streptomycin	500 µg/mL
Ampicillin	4 µg/mL
Erythromycin*	8 µg/mL, 16 µg/mL

*Multiple concentrations (in different plates) were used for erythromycin.

3.8 Whole genome sequencing: quality control and assembly

Whole genome sequencing was performed on select isolates as part of the work of Chapters 5 and 7. While the specific analytical methods used on these sequences are described in these chapters, all sequences underwent quality control checks and pre-processing regimes before the main analyses were performed. These are detailed below.

3.8.1 Quality check with FASTQC

Sequences obtained from whole genome sequencing were uploaded to Galaxy/mGVL 0.10-2 and analysed for quality using FastQC (Babraham Bioinformatics, Cambridge). Quality was assessed through examination of the per base sequence quality report. Quality was considered acceptable if the mean quality scores fell within the "good" range on the plot (represented by the green zones on the plot) (see Figure 3.1).

3.8.2 Sequence manipulation

Sequences were subsequently converted and trimmed using the FASTQ Groomer and Trimmomatic tools through Galaxy/mGVL 0.10-2. When trimmed, sliding window trimming was used, using 4 bases to average across and an average quality of 20 as the threshold. Groomed and trimmed sequences were once again analysed for quality with FastQC as above.

3.8.3 Sequence assembly

Groomed and trimmed sequences which passed the quality checks were assembled using the SPAdes genome assembler in Galaxy, generating a single fasta file for each isolate.

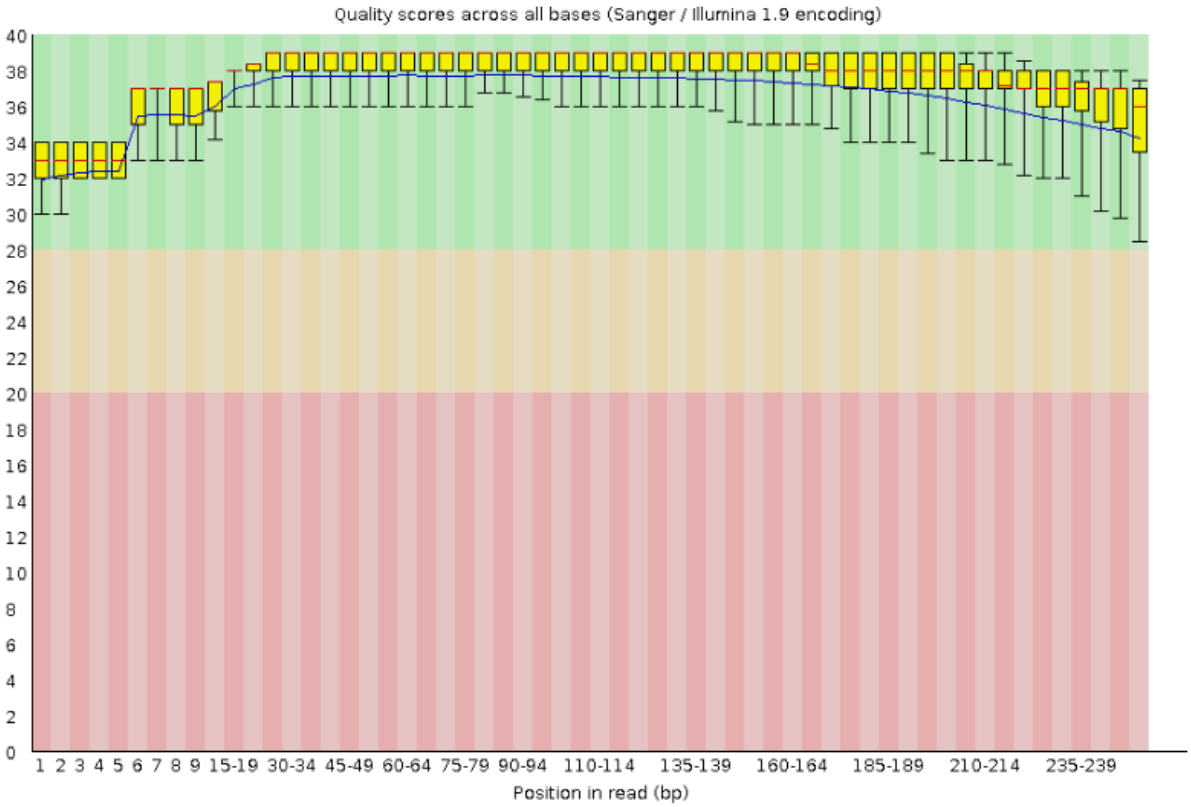

Figure 3.1: Example per base sequence quality plot. Mean is represented by the blue line. The red lines represent the median quality values at each position. The yellow box plots represent the inter-quartile range and the whiskers represent the 10% and 90% points. Good quality is represented by the green area at the top of the plot; the red area represents poor quality.

Chapter 4: Acquired macrolide resistance genes in nontypeable *Haemophilus influenzae*?

4.1 Introduction

Macrolides such as erythromycin and azithromycin are antibiotics that target the 23S subunit of the bacterial ribosome and act as protein synthesis inhibitors. Macrolides have been indicated for use in a number of conditions involving chronic respiratory bacterial infections, including chronic obstructive pulmonary disease (COPD). Many of these infections can involve NTHi and this has resulted in the emergence of macrolide resistant NTHi isolates within communities.

The activity of macrolides against NTHi isolates is limited in comparison to that against other species (azithromycin MICs typically cover the range of 0.25-4 µg/mL), although *in vitro* susceptibility of NTHi to macrolides is still relatively good.[252] This higher baseline MIC range has been attributed to the presence of an intrinsic efflux pump that is homologous to the *acrRAB* complex found in *E.coli*.[250] Additional high level resistance (azithromycin MICs typically ≥64 µg/mL) is relatively uncommon in NTHi but has increasingly been reported in some isolates. These instances have typically been associated with L4 and L22 ribosomal protein mutations and 23S rRNA mutations.[90,252]

A large range of acquired macrolide resistance genes (AMRGs) have been described in a wide variety of Gram-positive and Gram-negative bacteria. These include various *erm* genes that code for rRNA methylases that alter the 23S rRNA binding site of the macrolide to inhibit macrolide binding; various *mef* or related genes that encode alternate efflux pumps; and various others genes encoding macrolide inactivating enzymes.[1] These AMRGs have not been widely reported among NTHi isolates. However, there has been one significant report

of macrolide resistance genes by Roberts et al..[93] In that study, 106 isolates of NTHi from cystic fibrosis (CF) patients in a placebo-controlled trial of azithromycin were tested for the presence of *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A) by molecular methods. All isolates, including those on placebo, were reported to have one or more of the acquired macrolide resistance genes, but there was no clear association between the presence of these genes and the azithromycin or erythromycin MICs.[93] The only other report of an acquired macrolide resistance gene in *Haemophilus* spp. was *mef*(A) detected by microarray and sequencing in an isolate of *Haemophilus parainfluenzae*; in this case, macrolide MICs were >256 µg/mL.[257]

The aim of this study was to determine whether the presence of these AMRGs is widespread in the broader NTHi population.

4.2 Methods and Materials

4.2.1 Bacterial strains

The isolate collection for this study consisted of 172 clinical respiratory isolates of NTHi identified by colony morphology, X and V factor dependence and a positive PCR for either *fucK* or *hpd* as previously described.[271] Of these, 59 isolates were from CF patients, 27 isolates were from a cohort of non-CF bronchiectasis patients with significant prior macrolide use,[272] and the remaining 86 isolates were from a range of other patients. All isolates were cultured on chocolate agar.

4.2.2 Susceptibility testing

Azithromycin MICs were determined using Etest on MH-F media (see Chapter 3) and incubated at 37°C in 5% CO2 for 24 h using *H. influenzae* ATCC 49247 as a control. EUCAST breakpoints (susceptible, ≤0.12 µg/mL; resistant, >4 µg/mL) were used for interpretation.[69]

4.2.3 Detection of acquired macrolide resistance genes

Genomic DNA extracted using the ISOLATE II kit (Bioline, NSW, Australia) was used as template for PCR. All isolates were initially tested for the presence of the genes using locked nucleic acid dual-labelled hydrolysis probes (Sigma–Aldrich, NSW, Australia) in two quadruplex reactions for *erm*(A), *erm*(B) and *erm*(C) in reaction 1 and *erm*(F), *mef*(A) and *mef*(E) in reaction 2, with both reactions also including a 16S rRNA amplification control. Reaction conditions were as follows: iQ Multiplex Powermix (Bio-Rad, NSW, Australia) with probe and primers at final concentrations of 0.25 and 0.5 mM, respectively, over 30 cycles with an annealing temperature of 60°C. Primer sequences are given in Table 4.1.

Table 4.1: Probes and primers for detection of acquired macrolide resistance genes.

Primer or probe	Sequence[a]	Position[b]	GenBank accession[c]
16S probe	ttcCtcCacAtcTctacgc	697–715	L42023
16S F	ATTTCAGACTGGGTAACTAGAG	634–655	
16S R	GTCAGTACATTCCCAAGGG	735–753	
ermA probe	tcaCttGacAtaAgcctcc	184–202	X03216
ermA F	ACAAGACAACGTAATAGAAATCG	90–112	
ermA R	CTTTTATATTCTCAGAGGGGTTTAC	214–238	
ermB probe	ccgCcaTacCacAgat	528–543	M11180
ermB F	TCATCCTAAACCAAAAGTAAACAG	486–509	
ermB R	TTAGTAAACAGTTGACGATATTCTC	599–623	
ermC probe	agcAaaCccGtaTtccac	379–396	NC001395
ermC F	AGTACAGATATAATACGCAAAATTG	319–343	
ermC R	ATGCCAATGAGCGTTTTG	413–430	
ermF probe	cacCgcCaaCtgTcaa	512–527	AF219231
ermF F	TTGAAACTTGTCTATGAGGTAGG	472–494	
ermF R	CTCTAACAGACAGGAAATAAATGC	595–618	
mefA probe	tagTaaGcaCcgAaccag	260–277	U70055
mefA F	GGATCGTCATGATAGGAAGAAG	204–225	
mefA R	CAGGTAGCTCCATATAGAATGC	283–304	
mefE probe	tctCagAacCacAactcct	630–648	U83667
mefE F	CCTAAGCTGGGTAATCAAGTG	571–591	
mefE R	CTAAGAGTAATAAGGCAAACAATCC	658–682	

[a]Sequences are given 5′ to 3′ and, for probes, capital letters indicate locked nucleotides.
[b]Numbering based on ORF, except for 16S, which is based on a 1539 bp sequence.
[c]Reference sequences used for primer design.

Subsequently, the isolates were retested for the same genes (excluding *mef*(E)) using the primers and annealing temperatures described by Roberts et al.,[93] but on a real-time PCR platform using SYBR Green (Bio-Rad, NSW, Australia). Positives were checked on an agarose gel and amplicons of an appropriate size were sequenced using the respective amplification primers. The primer sequences are given in Table 4.2.

The control material was as follows: clinical isolates for *mef*(E), *mef*(A), *erm*(A) and *erm*(B) with gene identity confirmed by sequencing; pE194 for *erm*(C); and an *erm*(F) amplicon kindly provided by Zhongtang Yu (Department of Animal Sciences, Ohio State University, Columbus, OH, USA).

4.2.4 Detection of L4, L22 and 23S rRNA mutations

Any isolates exhibiting high-level macrolide resistance (azithromycin MIC > 64 µg/mL) underwent sequencing of the L4 and L22 protein genes and the 23S rRNA gene for the detection of macrolide resistance-associated mutations as previously described.[90]

Table 4.2: Primers used for detection of acquired macrolide resistance genes.*

Primer	Sequence (5' – 3')
ermA-F	GAA ATY GGR TCA GGA AAA GG
ermA-R	AAY AGY AAA CCY AAA GCT C
ermB-F	GAT ACC GTT TAC GAA ATT GG
ermB-R	GAA TCG AGA CTT GAG TGT GC
ermC-F	TCA AAA CAT AAT ATA GAT AAA
ermC-R	GCT AAT ATT GTT TAA ATC GTC AAT
ermF-F	CGA CAC AGC TTT GGT TGA AC
ermF-R	GGA CCT ACC TCA TAG ACA AG
mefA-MF6	GGA CCT GCC ATT GGT GTG
mefA-MF4	ACC GAT TCT ATC AGC AAA G

* As described by Roberts *et al*., 2011

4.3 Results and Discussion

The azithromycin MICs ranged from 0.09 to >256 µg/mL, with MIC50 and MIC90 values of 1.5 and 3 µg/mL, respectively. Two (1.2%) isolates were susceptible, 163 (94.8%) intermediate and 7 (4%) resistant according to EUCAST breakpoints (susceptible, ≤0.12 µg/mL; resistant, >4 µg/mL) (see Figure 4.1).[93]

Of the resistant isolates, five were low level (6–16 µg/mL) and were not further investigated, but two, isolates Ci115 and M3, had azithromycin MICs of >256 µg/mL and were further investigated by sequencing of the L4 and L22 ribosomal protein and 23S rRNA genes. One strain, isolate M3, had an A2058 mutation in the 23S rRNA gene and the other, isolate Ci115, had C2611T and R88P mutations in the 23S rRNA and L22 ribosomal protein genes, respectively, all of which have been previously associated with macrolide resistance (see Figures 4.2 and 4.3).[90,273,274] M3 carried additional alterations in 23S rRNA (see Table 4.3), although the significance of these alterations in regards to macrolide resistance in unclear. No alterations were detected in the L4 ribosomal protein in either isolate.

None of the acquired macrolide resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*(A) or *mef*(E) were detected in any of the isolates. The absence of the acquired macrolide resistance genes tested for in our isolates is in stark contrast to the findings of Roberts et al.,[93] where at least one gene was detected in each of their 106 isolates, two different genes were detected in 28 isolates and three different genes were detected in 7 isolates. This difference cannot be readily explained. Admittedly, only a small proportion of our isolates (27/172) came from a known background of high cohort macrolide exposure, but the Roberts et al.[93] study found the acquired macrolide resistance genes in isolates from both placebo and macrolide treatment groups, so recent macrolide use was not a prerequisite for the presence of the genes.

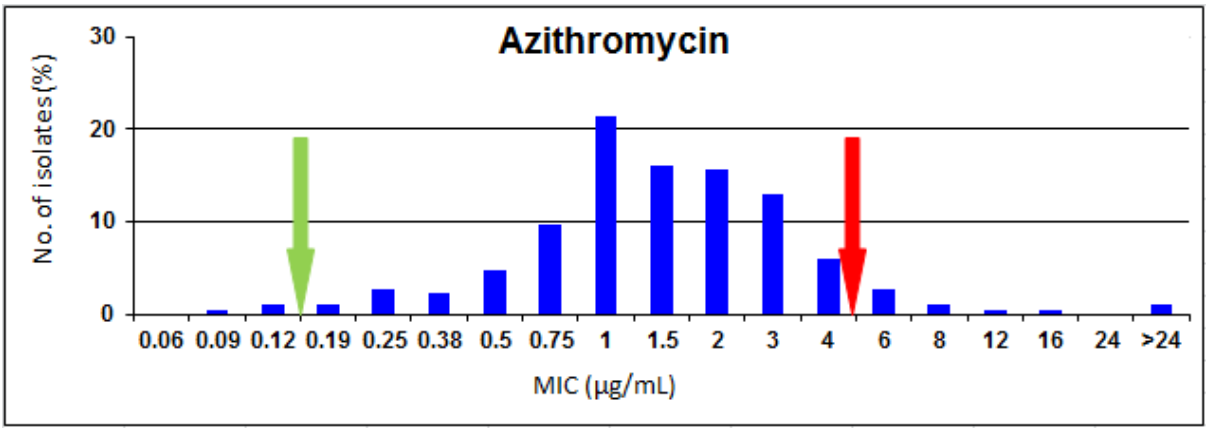

Figure 4.1: Distribution of azithromycin MICs of the isolates included in this study. The green arrow indicates the susceptible breakpoint and the red arrow indicates the resistant breakpoint. The majority of MICs fall within the intermediate breakpoint range (EUCAST breakpoints) and roughly follows a normal distribution, a finding typical of *H. influenzae*.

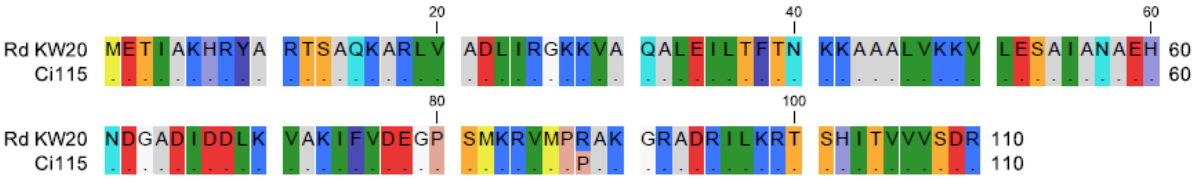

Figure 4.2: Sequence of L22 of *H. influenzae* Rd KW20 and Ci115. Matching residues are shown with dots. Ci115 carried a R88P alteration which has previously been associated with decreased macrolide susceptibility.

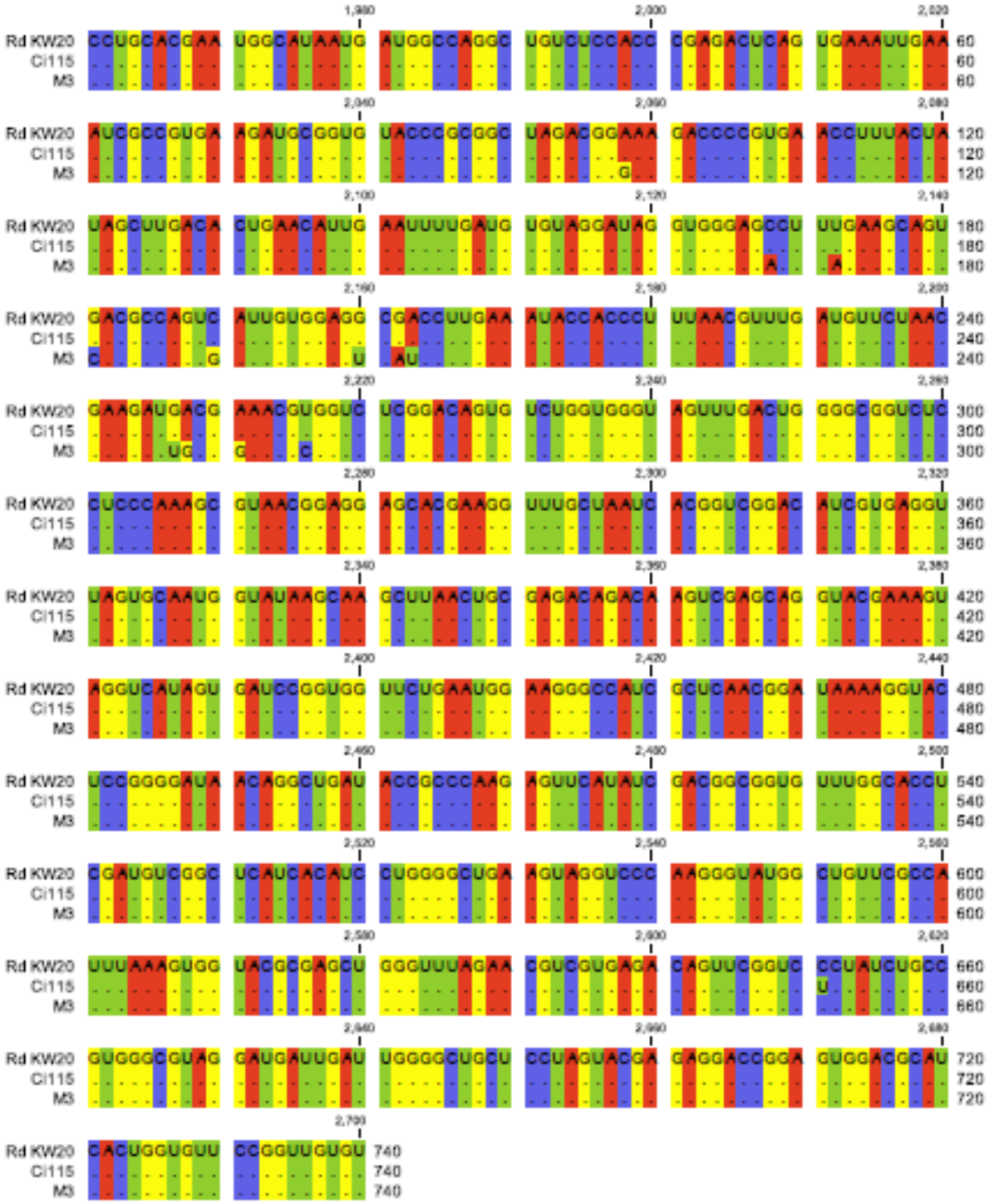

Figure 4.3: Sequence of 23S rRNA of *H. influenzae* Rd KW20, Ci115 and M3. Matching residues are shown with dots. Ci115 carried a C2611U alteration and M3 carried an A2058G alteration, both of which have previously been associated with decreased macrolide susceptibility. Additional alterations in 23S rRNA from Rd KW20 were also apparent but the relevance of these is not known.

Table 4.3: Complete list of L4, L22 and 23S rRNA alterations detected in Ci115 and M3.

Isolate	L4 alterations	L22 alterations	23S rRNA alterations
Ci115	None	R88P	C2611U
M3	None	None	A2058G, C2128A, U2132A, G2141C, C2150G, G2160U, G2162A, A2163U, G2207U, A2208G, A2211G, U2216C

Similarly, the isolates from the Roberts et al.[93] study were more resistant than ours, with 27/106 (25%) resistant, but again the acquired macrolide resistance genes were detected

across all isolates irrespective of MIC, including *erm*(B) and *mef*(A) in the single susceptible isolate.

To investigate technical differences, we retested our isolates using the primers used in the Roberts et al.[93] study and found 21 isolates to be presumptively positive for *mef*(A) and 6 for *erm*(A). The azithromycin MIC range for these isolates was 0.125 to 256 µg/mL with MIC50 and MIC90 values of 1.5 and 4 µg/mL, respectively. In the additional investigation detailed below, these positive PCR results were found to be false positives.

The amplicons from the *mef*(A) and *erm*(A) primers were difficult to distinguish from the 942 and 332 bp amplicons of the respective positive controls on gel electrophoresis (see Figures 4.4 and 4.5). They were subsequently sequenced and identified using a BLASTn search. The false positive *mef*(A) amplicon was found to span nucleotides 588138–589053 (916 bp) encompassing the transcription accessory protein (*TAP*) and adjacent to the DNA gyrase B gene (*gyrB*) in *H. influenzae* Rd KW20 (GenBank accession L42023). The false positive *erm*(A) amplicon was found to span nucleotides 32743–32383 (361 bp) encompassing the rod shape-determining protein (*ROD*), also in *H. influenzae* Rd KW20. Similar sequences are found in Genbank sequences from other strains of *H. influenzae*. Close examination of the ends of these sequences revealed significant degrees of similarity to the primers, particularly at the 3′ end. The degree of similarity differed depending on the particular strain and this is briefly illustrated in Table 4.4.

These observations raise the question as to why all of our isolates did not give false positives with these PCRs, given that these genes are part of the core genome of *H. influenzae*. When we examined the relevant regions of GenBank sequences for these genes in other strains of *H.*

Figure 4.4: Electrophoresis gel of amplicons produced by the Roberts primers for the detection of *mef*(A). The false positive amplicons (indicated by the red box) were difficult to distinguish from a true positive amplicon (indicated by the green box) by gel electrophoresis alone due to their similar size.

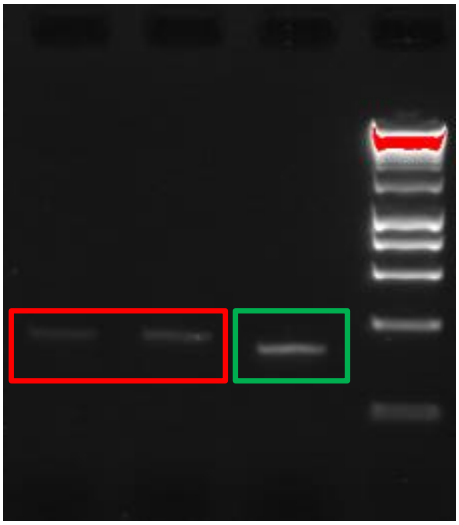

Figure 4.5: Electrophoresis gel of amplicons produced by the Roberts primers for the detection of *erm*(A). The false positive amplicons (indicated by the red box) were difficult to distinguish from a true positive amplicon (indicated by the green box) by gel electrophoresis alone due to their similar size.

Table 4.4: Example sequences showing deduced primer binding sites for false positive amplicons.

	Details	Sequence
erm(A)	Rd* 32743-23	TGGTGGATTAAGCGGAAAAGG
	KR494# 1558106-126	TGGTGGATTAAGCGGAAAAGG
	Primer ermA-F (5' -3')	GAAATYGGRTCAGGAAAAGG
	Rd 32401-383 (complimentary)	TTCAAAACCAAATGACTAC
	KR494 1557730 to 1557746 (complimentary)	CTCGAAACCAAATGACTAC
	Primer ermA-R **(3' – 5')**	CTCGAAAYCCAAAYGAYAA
mef(A)	Rd 588138-56	TTTAGTGCATCAGGCAAAG
	KR494 216063-216080	TTTAACGCATCAGCAAAG
	Primer mefA-MF4 (5'-3')	ACCGATTCTATCAGCAAAG
	Rd 589037-53 (complimentary)	GTGGTTACCGAAAGCCA
	KR494 216961-216977 (complimentary)	GTGGTTACCGAAAACCA
	Primer mefA-MF6 **(3'-5')**	GTGTGGTTACCGTCCAGG

**H. influenzae* Rd (GenBank accession L42023) and # *H. influenzae* KR494 (Genbank accession CP005967)

influenzae, some sequence variation that could affect primer binding was evident. In addition, our false positive reactions had Ct values of between 24 and 27 cycles compared with 15–20 cycles for the positive controls, so it would not be unexpected for small variations

in nucleotide sequence or DNA quality across our test isolates to result in inconsistent amplification of these chromosomal targets. No further analysis was performed on the *erm*(A) false positive amplicons, but in an attempt to determine why only some of our isolates were producing false positive amplicons using the Roberts *mef* primers, we designed a number of primer sets to investigate the presence of any genome variations between isolates producing the false positive amplicon and isolates that produced correctly negative results. Firstly, we designed primers around 200 bp outside of where the false positive binding was occurring. These primers were designed to show any sequence differences in the region generating the false positive amplicons that may have affected the binding affinity of the Roberts primers. We also designed primers amplifying both *gyrB* (an ORF which flanks the region covered by the false positive amplicon) and *TAP* to determine if one or both of these genes were missing in our true negative isolates, and to further confirm the presence of any sequence differences in these genes between false positive isolates and true negative isolates. Finally, we designed primers to amplify the region bridging *gyrB* and *TAP*. The aim here was to determine if *gyrB* and *TAP* were within the same proximity of each other in false positive isolates, and if the proximity was different for true negative isolates. The primers were designed to bind within the last 300 bp of *gyrB* and the first 300 bp of *TAP*, and would only produce an amplicon if *gyrB* and *TAP* were within close proximity of each other; furthermore, any amplicon size differences would indicate differences in the region between these genes and might explain isolate differences using the Roberts primers. All of these primer sequences are listed in Table 4.5.

Table 4.5: Primer sequences for the investigation of the false positive binding of the Roberts *mef* primers.

Primer	Sequence	Purpose
F-FPMefA	TCC ACC ACT TCA AAT AAC	Amplification and sequencing of entire false positive amplicon
R-FPMefA	GTC CGT TTA TTC AGC TTC	
F-GyrB	GGA TCT TTT TCT TGG CAG T	Amplification of *gyrB*
R-GyrB	GGT TTG GTG GCG ATT ATT	
F-TAP	GTG TGG ATA AAT GGT GGT	Amplification of *TAP*
R-TAP	GAA AAA GCG GAA GAA GAA G	
F-bridge	CCG TTT TGG CGA TGC TTT TC	Amplification of the region bridging *gyrB* and *TAP*
R-bridge	CGT GGA AAT ATG CAG CGA GA	

For our investigations, we selected 5 isolates that produced the false positive amplicon with the Roberts *mef* primers and 5 isolates that did not produce this amplicon. Testing of these 10 isolates revealed that all of them carried *gyrB* and *TAP*, suggested that the discrepancy was

not caused by the absence of one or both of these genes in the true negative isolates. In addition, we performed agarose gel electrophoresis on the amplicons produced by the *gyrB* and *TAP* primers, as well as the amplicon produced by the bridge primers. The amplicons for all 3 primer sets were shown to be of the expected size regardless of whether the isolate being tested was a false positive amplicon producer or a true negative isolate. This indicated that the discrepancy was not due to a large scale insertion or deletion in *gyrB* or *TAP*, and that the length of the bridging region between these genes was similar enough to be indistinguishable on an agarose gel. We subsequently submitted the same 10 isolates for sequencing using the false positive amplicon primers described in Table 4.5. However, analysis of the sequences did not reveal any sequence differences between the false positive isolates and the true negative isolates that would explain why the amplicon was being produced with some isolates and not others.

In addition to the sequencing analysis described above, we also ran an annealing temperature gradient with the Roberts *mef* primers using two false positive isolates and two true negative isolates. The temperature gradient covered the range of 50-60°C and the PCR was continued for 40 cycles. At higher temperatures, the Ct values of the true negative isolates exceeded 30 cycles, and the Ct value did not drop below 30 cycles until an annealing temperature of 52°C. By contrast, the Ct values of the false positive isolates fell below 30 cycles at temperatures as high as 60°C. The Ct values of a false positive isolate (CF21) and a true negative isolate (CF11) are listed in Table 4.6 to demonstrate this contrast.

The various CF21 and CF11 amplicons were run on an agarose electrophoresis gel (see Figure 4.6). For CF11, an amplicon of similar size to the false positive amplicon produced

Table 4.6: Ct values of CF21 (false positive isolate) and CF11 (true negative isolate) from the temperature gradient using the Roberts' *mef* primers.

Annealing Temperature (°C)	CF21 Ct Values	CF11 Ct Values
60.0	24.54	36.94
59.6	23.66	35.41
58.3	23.26	34.76
56.4	21.84	31.43
53.9	20.87	30.20
52.1	20.46	26.90
50.8	19.69	25.34
50.0	19.01	23.72

by CF21 could be visualised across all temperatures, although at higher temperatures the amplicon was very faint. Given that no apparent sequence alterations in the region covered by the false positive amplicon (including in *gyrB* and *TAP*) were detected, it is therefore likely that the "true negative" isolates are capable of producing the same false positive

amplicon as the previously described false positive isolates. Given that the PCR used for the Roberts *mef* primers was originally run at 30 cycles using an annealing temperature of 56°C, it is unsurprising that these isolates were not detected during the original assays. It is still unclear why only some isolates produced this amplicon at the annealing temperature of 56°C, although it may perhaps be attributable to subtle variations in DNA concentration or quality between the starting extracted DNA material for each individual isolate.

In any case, the emergence of false positive amplicons using the Roberts *mef* and *erm*(A) primers in our study makes it interesting to speculate as to whether some of the positive results in the Roberts et al.[93] study, particularly for *mef*(A) and *erm*(A), could have been similar false positives. This might explain the large number of positives and the absence of associated raised macrolide MICs. However, although none of the amplicons in that study was confirmed by sequencing, they were confirmed using hybridization probes.

In conclusion, our study shows that acquired macrolide resistance genes are not widespread in NTHi and the high prevalence of these genes previously reported might be unique to the specific circumstances of that study. We also demonstrate that the use of primers in organisms other than those for which the primers were initially designed and evaluated can produce false positive amplification not easily detected without confirmatory tests such as sequencing.

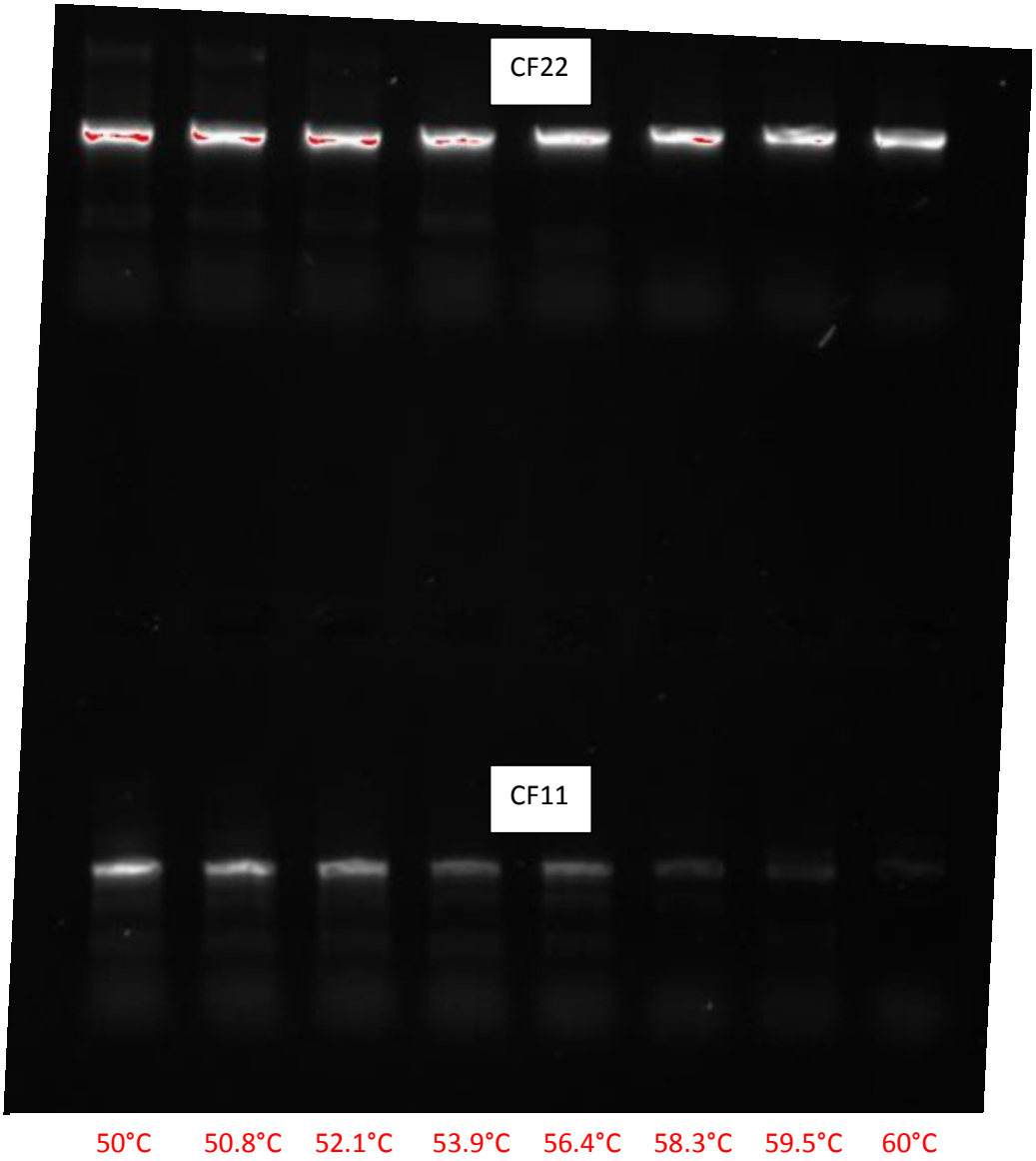

Figure 4.6: Electrophoresis gel of amplicons produced during temperature gradient with the Roberts primers for the detection of *mef*.

4.4 Further developments

The experimental work presented within this chapter was performed during the period of January 2014 – December 2014. The experimental design and result interpretation were guided by information we had at the time the work was performed; this included the

information presented by Roberts et al., as well as personal communications with Dr. Roberts regarding the design of the primers used in her manuscript.

The work presented here was peer reviewed and published in the Journal of Antimicrobial Chemotherapy in 2015.[275] Since the publication of this work, Roberts et al. have published a comment on our manuscript in response to some of our findings and interpretations.[276] Notably, Roberts et al. point out that the primers that we stated that they used in their original study were not the primers they actually used, but were a different set of primers from an unaffiliated study, and that therefore we are unable to speculate about the possibility of the presence of false positives among the positive findings reported in the study. Our group have also published a response to this comment.[277] The responses of Roberts et al. and our group can be found in Appendix 1.

Chapter 5: Utilisation of whole genome sequencing techniques in the investigation of acquired macrolide resistance genes in *Haemophilus influenzae*

5.1 Introduction

The use of whole genome sequencing is becoming more common as an alternative to other techniques for DNA analysis such as Sanger sequencing. Whole genome sequencing techniques can be utilised for the investigation of antibiotic resistance in bacteria and is more affordable than ever. Such techniques offer a number of analytical advantages over other techniques used to investigate resistance. For example, in addition to its use in characterising isolates for the presence of known resistance markers, whole genome sequencing can also be useful for detecting unknown potential contributors to resistance in isolates, assuming one has good knowledge of functional motifs. This can be particularly useful for resistant isolates that lack other previously described resistance determinants. In addition, whole genome sequencing also provides a more practical method to screen isolates for the presence of multiple resistance genes and determinants simultaneously.

The work described in this chapter encompasses a number of projects, but the overall aim was to utilise whole genome sequencing techniques to further investigate macrolide resistance determinants in NTHi. Firstly, we aimed to find further evidence of the presence of AMRGs in a range of NTHi isolates, including the isolates exhibiting high-level macrolide resistance detected in Chapter 4 (Ci115 and M3). This involved whole genome sequencing these isolates and screening them for the presence of a larger set of specific AMRGs, including each of the genes investigated in Chapter 4, using the previously described SPANDx pipeline.[278] We were additionally interested to see if we could detect these genes in

a wider range of NTHi isolates of unknown macrolide resistance phenotype, given that the study of Roberts et al. (2011) found AMRGs in isolates with a variety of resistance phenotypes,[93] and as such we expanded the analysis to include further isolates for which whole genome sequences are publically available.[279]

To supplement the above analysis, we also aimed to detect AMRGs in Ci115 and M3 by submitting these sequences to the Comprehensive Antibiotic Resistance Database (CARD).[280] Doing this would provide another avenue to screen for the presence of AMRGs in these isolates, including those not included in the initial SPANDx analysis. The sequences were also submitted to the Resistance Gene Identifier (RGI) within CARD[280] as an additional step in detecting macrolide resistance markers in Ci115 and M3.

While the main focus of this thesis is on the potential presence and role of AMRGs in NTHi, we initially decided to further investigate the cause of the macrolide resistance phenotype in Ci115 and M3 by extracting the sequences of the L4 and L22 ribosomal protein and 23S rRNA genes from the whole genome sequence output and analysing these for known mutations associated with macrolide resistance. While we had previously performed these analyses in Chapter 4 via Sanger sequencing, performing these additional analyses still had some value. Firstly, repeating analyses on L4, L22 and 23S rRNA served as a kind of control for quality of the analyses of the whole genome sequences in general, in the sense that it would be expected that alterations detected from the Sanger sequences in Chapter 4 would also be detected from the whole genome sequences in this chapter. Any conflicts found between the two approaches may imply errors in the analyses that would warrant further investigation, and may compromise any further work performed on the whole genome sequences related to AMRGs. In addition, analysis of the whole genome sequences could provide further information regarding the role of 23S rRNA in macrolide resistance. The primers used in Chapter 4 were designed to amplify domain V only and do not provide

coverage of domain II; whole genome sequencing can be used to provide this information. Furthermore, whole genome sequencing can also be used to determine how many of the 6 operons of 23S rRNA found in *H. influenzae* carry particular alterations (i.e. the dosage). In addition, very recent evidence not available at the time the work of Chapter 4 was completed has indicated that that alterations in the regulatory gene (*acrR*) of the acrAB system can result in overexpression of *acrB* and may play a role in macrolide resistance.[251] As such, we decided to use whole genome sequencing to attempt to detect alterations in this gene in our resistant isolates that could also be contributing to the resistant phenotype.

Finally, we attempted to detect the presence of any unknown contributors to macrolide resistance in Ci115 and M3 with transformation experiments on susceptible recipients using genomic DNA extracted from these isolates. This involved selecting and whole genome sequencing azithromycin-resistant transformants and comparing them to the sequences of the donors and recipients to determine whether specific sequences or snps had transferred across that may be contributing to macrolide resistance.

5.2 Methods and Materials

5.2.1 Bacterial strains and genomes

The primary strains of interest were 2 respiratory NTHi isolates (Ci115 and M3) that had previously been found to exhibit high-level macrolide resistance (azithromycin MIC ≥ 256 µg/mL) in Chapter 4 of this work. Sanger sequencing of both isolates in Chapter 4 had previously revealed that isolate M3 carried an A2058 mutation in the 23S rRNA gene and that isolate Ci115 carried C2611U and R88P mutations in the 23S rRNA and L22 ribosomal protein genes, respectively.

For the *in silico* AMRG search (see below), we chose to look more broadly for the presence of these genes in a wider collection of NTHi WGSs of unknown macrolide resistance phenotype. The strains included in this analysis were derived from the "De Chiara collection", for which whole genome sequences are publically available.[279] The complete set of genomes included in this study is listed in Table 5.1.

For transformation assays, the recipient strains were NTHi strain 86-028NP and *H. influenzae* Rd KW20.

5.2.2 Transformation assays

The transformation assays performed in this study were adapted from the methodology previously described by Ubukata et al. (2001)[281] and are described below.

5.2.2.1 Preparation of genomic DNA

Genomic DNA of Ci115 and M3 was prepared as previously described in Chapter 3.

5.2.2.2 Growth and washing of recipient cells

Recipient cells were grown overnight on chocolate agar and a heavy suspension (equivalent

Table 5.1: Complete list of genomes included in this study.

#*	Strain**	#*	Strain**	#*	Strain**
1	Ci115	**32**	Hi1621	**63**	Hi805
2	M3	**33**	Hi1622	**64**	Hi88
3	DC7331102	**34**	Hi1623	**65**	Hi968
4	Hi1008	**35**	Hi1630	**66**	Hi973
5	Hi11	**36**	Hi167	**67**	Hi981
6	Hi1124	**37**	Hi17	**68**	HiPittII
7	Hi1158	**38**	Hi176	**69**	HiR2846
8	Hi1180	**39**	Hi177	**70**	HiR3021
9	Hi1200	**40**	Hi199	**71**	RM600672
10	Hi1207	**41**	Hi2019	**72**	RM601173
11	Hi1231	**42**	Hi206	**73**	RM601974
12	Hi1233	**43**	Hi24	**74**	RM603375
13	Hi1247	**44**	Hi264	**75**	RM605177
14	Hi1268	**45**	Hi285	**76**	RM701878
15	Hi1363	**46**	Hi375	**77**	RM702879
16	Hi1500	**47**	Hi398	**78**	RM702980
17	Hi1513	**48**	Hi432	**79**	RM706883
18	Hi1549	**49**	Hi443	**80**	RM712284
19	Hi1553	**50**	Hi477	**81**	RM730885
20	Hi1556	**51**	Hi486	**82**	RM730986
21	Hi1557	**52**	Hi492	**83**	RM734787
22	Hi1558	**53**	Hi525	**84**	RM744888
23	Hi1559	**54**	Hi609	**85**	RM745989
24	Hi1560	**55**	Hi639	**86**	RM746590
25	Hi1566	**56**	Hi658	**87**	RM747791
26	Hi1568	**57**	Hi667	**88**	RM749092
27	Hi16	**58**	Hi709	**89**	RM761793
28	Hi1606	**59**	Hi723	**90**	RM763794
29	Hi1607	**60**	Hi740	**91**	RM787695
30	Hi1619	**61**	Hi787	**92**	86-028NP
31	Hi162	**62**	Hi794		

*Each genome was assigned a number for convenience (for referring to specific strains later in the study).
**Ci115 and M3 are respiratory strains with high-level macrolide resistance that were isolated during the study described in Chapter 4. The remaining sequences were sourced from the De Chiara collection.[279]

to McFarland standard of 2.0) of each strain was prepared in 2.5 mL saline. This was added to 100 mL of sBHI and incubated in air at 37°C on a shaker (180 rpm) until reaching early- to mid-log phase growth (represented by an absorbance of approximately 0.5 at 650 nm). The cells were chilled on ice for 30 minutes and subsequently split into two 50 mL Falcon tubes. Cells were centrifuged at 4200 g and 4°C for 20 min, and the supernatant was discarded. Cells were then resuspended and washed four times in 50 mL cold PSG buffer (15% glycerol, 272 mM sucrose, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4) using the same centrifugation conditions as above, and recombining the cells into 1 Falcon tube after the second wash. After the final wash, cells were resuspended in 1 mL of cold PSG.

5.2.2.3 Transformation of NTHi 86-028NP and Haemophilus influenzae Rd KW20

Transformation of the washed recipient cells was performed by electroporation using the Bio-Rad MicroPulser^{TM}. 40 µL of cells was combined and mixed gently with 5 µL of donor genomic DNA in a cold 1.5 mL microfuge tube and incubated on ice for 1 minute. The mixture was then transferred to a chilled 2 mm cuvette and pulsed using the EC2 setting of the MicroPulser (V=2.5kV, time constant = 5 ms).

5.2.2.4 Growth of transformants

Transformed cells were flushed from the cuvette using pre-warmed sBHI. The cells were then incubated in 1 mL pre-warmed sBHI in air at 37°C on a shaker (180 rpm) for 4 hours.

After growth, cells were plated out (100 µL in varying dilutions) onto chocolate agar supplemented with 16 µg/mL azithromycin. To ensure the agar was suitably selective and that the probability of random mutants emerging was low, donor and recipient cells were also cultured using these plates. In addition, donor and recipient cells were suspended in saline to a 0.5 McF standard; donor suspensions were diluted with recipient suspensions (in varying

dilutions) and these were cultured on the agar to ensure that transformants could be isolated from a mix with non-resistant cells.

After growth, potential transformants were procured for further examination.

5.2.2.5 Antibiotic susceptibility testing

Azithromycin MICs of transformants were determined using Etest on MH-F agar (refer to Chapter 3 for further information) and incubated at 378C in 5% CO2 for 24 h using *H. influenzae* ATCC 49247 as a control.

5.2.3 Whole genome sequencing

Isolates of interest were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for whole genome sequencing. Isolates submitted were M3 and Ci115, NTHi 86-028NP, and select transformants (see Table 5.2 for list of transformants submitted).

5.2.4 Sequence quality check and assembly

Sequences obtained from whole genome sequencing were uploaded to Galaxy/mGVL 0.10-2 and analysed for quality and assembled as previously described in Chapter 3 of this thesis. The NTHi 86-028NP contained 237 separate Contigs ranging from 56 to 245848 nucleotides in length, the Ci115 file contained 334 separate Contigs ranging from 56 to 257287 nucleotides in length, and the M3 file contained 250 Contigs ranging from 56 to 225265 nucleotides in length.

5.2.5 Extraction of *acrR* and the L4 and L22 gene sequences

Each assembled sequence was submitted to Rapid Detection using Subsystem Technology (RAST, http://rast.nmpdr.org/) for annotation, and sequences for L4, L22 and *acrR* were extracted from the resulting excel spreadsheet outputs. Each extracted sequence was saved as

Table 5.2: List of recovered transformants with azithromycin MIC values.

Transformant	Donor	Recipient	Azithromycin MIC (µg/mL)
CA1	Ci115	NTHi 86-028NP	64
CA3	Ci115	NTHi 86-028NP	32
CA4	Ci115	*H. influenzae* Rd KW20	96
CA6	Ci115	*H. influenzae* Rd KW20	24

a fasta file and imported into CLC Main Workbench 7. The sequences of M3 and Ci115 were assembled against the sequences of NTHi 86-028NP to generate a Contig file for the detection of sequence variations. All sequences were translated to proteins and the protein sequences of Ci115 and M3 were aligned against the protein sequence of NTHi 86-028NP to detect alterations in protein sequence and length.

5.2.6 Extraction of 23S rRNA sequences

The assembled sequences of M3 and Ci115 were mapped to the NTHi 86-028NP 23S sequence (NC_007146) using the Burrows-Wheeler-Aligner optimised for long reads (BWA-MEM) in Galaxy. The resulting bam and bai files were retrieved and the bam file was opened with the Integrative Genomics Viewer (IGV) to visualise alterations in the 23S sequence. The dosage of each alteration was estimated by checking the percentage of 23S reads containing the alteration (see Table 5.3).

5.2.7 In silico AMRG search

A panel of known acquired MLS_B resistance genes was developed (see Table 5.4). The selection of genes for this analysis was guided by the list of recognised genes in the Roberts database.[95]

The analysis was performed using the SPANDx pipeline (Sarovich 2014).[278] The nucleic acid sequences of genomes of interest were converted to amino acid sequences and the tBLASTn algorithm was used to assess amino acid identity as previously described and recommended by Derek Sarovich (Sarovich et al. 2016).[282] A BLAST score ratio of 0.70 was used as a cut-off; genes with scores below this cut-off were considered absent from the WGSs.

Table 5.3: Extrapolation between read % output from IGV and copy number.

Approximate % of reads carrying alteration	Number of copies carrying alteration
16-17%	1
33-34%	2
50%	3
66-67%	4
83-84%	5
100%	6

Table 5.4: List of genes included in the AMRG screen.

erm		**Efflux**		**Deactivation**	
Gene	**Genbank #**	**Gene**	**Genbank #**	**Gene**	**Genbank #**
erm(A)	X03216	*car*(A)	M80346	*ere*(A)	M11277
erm(B)	EF450709	*lmr*(A)	X59926	*ere*(B)	A15097
erm(C)	V01278	*lsa*(B)	AJ579365	*lnu*(A)	M14039
erm(D)	M29832	*lsa*(C)	HM990671	*lnu*(B)	AJ238249
erm(E)	X51891	*lsa*(E)	AF408195	*lnu*(C)	AY928180
erm(F)	M14730	*mef*(A)	U70055	*lnu*(D)	EF452177
erm(G)	M15332	*mef*(E)	AF376746	*lnu*(E)	KF287643
erm(H)	M16503	*msr*(A)	X52085	*lnu*(F)	EU118119
erm(N)	X97721	*msr*(C)	AY004350	*mph*(A)	D16251
erm(O)	M74717	*msr*(D)	AF227521	*mph*(B)	D85892
erm(Q)	L22689	*msr*(E)	FR751518	*mph*(C)	AB013298
erm(R)	AY623658	*ole*(B)	L36601	*mph*(E)	FR751518
erm(S)	M19269	*ole*(C)	L06249	*mph*(F)	AM260957
erm(T)	M64090	*sal*(A)	KC693025	*vat*(A)	L07778
erm(V)	U59450	*srm*(B)	X63451	*vat*(B)	U19459
erm(W)	286053	*tlr*(C)	M57437	*vat*(C)	AF015628
erm(X)	M36726	*vga*(A)	M90056	*vat*(D)	L12033
erm(Y)	AB014481	*vga*(B)	U82085	*vat*(E)	AF139725
erm(31)	AF079138	*vga*(C)	NC_013034	*vgb*(A)	M20129
erm(32)	AJ009971	*vga*(D)	GQ205627	*vgb*(B)	AF015628
erm(33)	AJ313523	*vga*(E)	FR772051		
erm(34)	AY234334				
erm(35)	AF319779				
erm(36)	AF462611				
erm(37)	Z74025				
erm(38)	AY154657				
erm(39)	AY487229				
erm(40)	AY570506				
erm(41)	EU177504				
erm(42)	FR734406				
erm(43)	HE650138				

5.2.8 AMRG search in CARD

To more thoroughly search for AMRGs in our resistant isolates, the WGSs of Ci115 and M3 were submitted to CARD.[280] A BLASTn search was performed on the Contigs against the complete database of resistance genes. Only Contigs that were ≥250 nucleotides in length were included in this analysis; 97 Contigs from Ci115 and 60 Contigs from M3 were ultimately submitted using this criterion. In addition, fasta files for both isolates were submitted to the Resistance Gene Identifier (RGI) in CARD[280] for annotation to detect any other predicted macrolide resistance-associated gene products.

5.3 Results and Discussion

Analysis of the L4 and L22 sequences extracted from the whole genome sequencing output for isolates Ci115 and M3 confirmed the findings reported in Chapter 4. Ci115 carried an R88P alteration in L22 while M3 had no alterations in L22. Neither of these isolates carried alterations in L4. Analysis of *acrR* (see Figure 5.1) in M3 revealed a complete gene that also bore a number of nucleotide substitutions. The *acrR* gene in Ci115 lacked these substitutions, but bore an INS 504A alteration that resulted in a frame shift and the generation of an early stop codon. The resulting protein was 170 amino acids in length, representing an incomplete protein (complete protein is 188 amino acids in length). It is not entirely clear how much these variations contribute to macrolide resistance in this instance. Seyama et al. (2016a)[251] have very recently reported that insertions and deletions within *acrR* resulting in the generation of an incomplete protein can resulting in overexpression of *acrB*, which in turn results in increased clarithromycin resistance (but with a comparatively smaller effect on azithromycin resistance). While the 504A insertion seen in Ci115 was not reported in the Seyama et al. study,[251] the varied nature of the insertions and deletions reported in that study indicate that *acrR* serves as a hotspot for the development of macrolide resistance and it is possible that INS 504A in *acrR* may also be a contributing factor here. However, it is not possible to quantify its effect on phenotype in Ci115 given that this strain also carries other alterations associated with macrolide resistance, and further work would be required to transform this altered *acrR* variant into another *H. influenzae* strain in isolation to observe its effect in isolation. In the Seyama et al. study,[251] a clarithromycin-susceptible isolate was reported to have a complete acrR protein with multiple substitutions (using *H. influenzae* Rd KW20 as the reference) much like M3, although the specific alterations were not reported. We therefore speculate that the variations observed in *acrR* in M3 are unlikely to be having an effect on macrolide resistance.

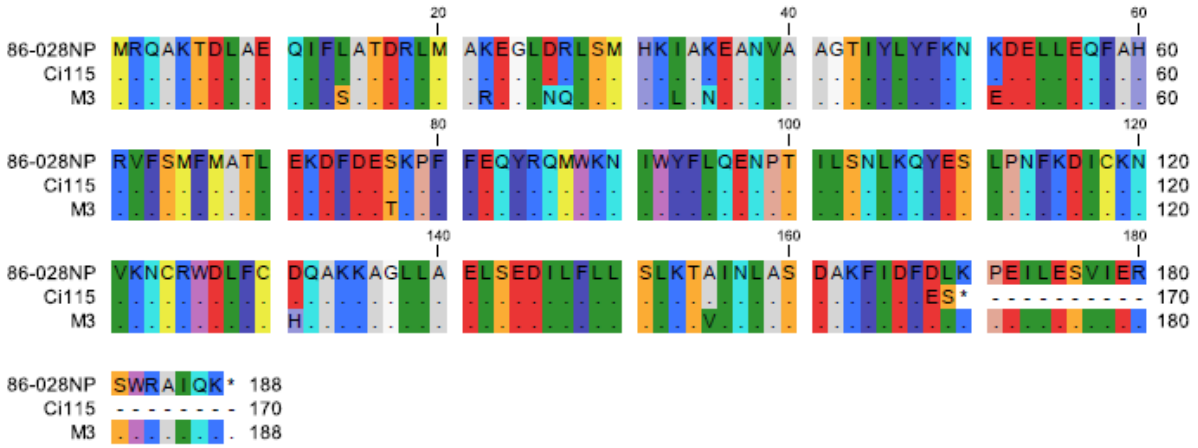

Figure 5.1: Sequences of acrR of Ci115 and M3 aligned against NTHi 86-028NP. Matching residues are shown with dots.

Alterations detected in 23S rRNA of Ci115 and M3 are summarised in Table 5.5. All alterations detected in Chapter 4 were also detected here (highlighted in yellow in Table 5.5), including the known macrolide resistance-associated alterations C2611U in Ci115, and A2058G and G2160U in M3. Interestingly, while all 6 operons of 23S rRNA carried the C2611U alteration in Ci115, it appeared that only 5 of the 6 operons of 23S rRNA carried the A2058G alteration in M3, indicating that it is not necessary for all operons to carry the A2058G alteration for azithromycin resistance to develop in a strain of NTHi. To our knowledge, this is the first time that any attempt has been made to examine the dosage in 23S rRNA in *H. influenzae* in the context of decreased macrolide susceptibility. However, further work would be required to demonstrate the precise effect of dosage of various 23S rRNA mutations on macrolide resistance in NTHi, in isolation from other alterations also known to be associated with macrolide resistance.

A number of other alterations to the 23S rRNA that had not been detected in Chapter 4 were also found. Both isolates carried a number of alterations in domain II (these had not been previously investigated in Chapter 4 and are highlighted in green in Table 5.5), but neither isolate bore an alteration at position 754, a known ribosomal mutation hotspot in the development of macrolide resistance,[274] and the remaining alterations fall outside the range covered by hairpin 35. M3 additionally carried alterations at positions 2216 and 2579 in domain V (highlighted in orange in Table 5.5) although these too have not been previously reported to be associated with resistance. The significance of these alterations in regards to macrolide resistance is unclear and beyond the scope of this work.

Attempts to transform NTHi 86-028NP and *H. influenzae* Rd KW20 with genomic DNA from Ci115 resulted in the generation of only a small number of colonies (2 from experiments using NTHi 86-028NP as the recipient and 2 using *H. influenzae* Rd KW20) and these were subsequently investigated using whole genome sequencing. None of the colonies

Table 5.5: Complete list of 23S rRNA alterations* detected in Ci115 and M3.

Ci115		M3					
23S rRNA alteration**	**Number of operons with alteration**	**23S rRNA alteration****	**Number of operons with alteration**	**23S rRNA alteration****	**Number of operons with alteration**	**23S rRNA alteration****	**Number of operons with alteration**
U538A	6/6	C93U	5/6	U1165G	6/6	C1736U	6/6
C539A	6/6	A198C	6/6	U1210A	6/6	C1865A	6/6
A690G	1/6	C503U	6/6	A1218C	6/6	C1870A	6/6
A819G	1/6	G509A	6/6	U1229G	6/6	**A2058G**	**5/6**
G1087C	6/6	U544A	6/6	A1230G	6/6	**C2128A**	**6/6**
C1088A	6/6	C545A	6/6	C1290U	3/6	**U2132A**	**6/6**
A1090G	6/6	U546G	6/6	C1506U	5/6	**G2141C**	**6/6**
G1106A	6/6	G1098A	3/6	U1507A	5/6	**C2150G**	**6/6**
G1146C	6/6	G1106U	6/6	U1514A	5/6	**G2160U**	**6/6**
U1150C	6/6	G1145C	3/6	C1710U	6/6	**G2162A**	**6/6**
U1153A	6/6	G1146C***	3/6	G1711U	6/6	**A2163U**	**6/6**
		G1146A***	3/6				
A1159G	6/6	U1147C	6/6	G1712A	6/6	**G2207U**	**6/6**
U1165G	6/6	U1150C	3/6	A1730U	6/6	**A2208G**	**6/6**
U1210A	6/6	U1153A	3/6	G1731A	6/6	**A2211G**	**6/6**
A1218C	6/6	A1159G	3/6	G1735A	6/6	**U2216C**	**6/6**
A1229G	6/6					U2579A	6/6
A1230G	6/6						
A1542G	1/6						
C2611U	**6/6**						

*Alterations compared to NTHI 86-028NP.
**Alterations previously recorded in Chapter 4 are highlighted in red text.
*** G1146 of M3 was altered in all copies of 23S rRNA; 3 copies bore G1146C and 3 copies bore G1146A.

recovered from this experiment had azithromycin MIC values as high as the original donor (≥256 μg/mL), with MICs falling in the range of 24-96 μg/mL (see Table 5.2). At this point of the analysis, this indicated that either the recovered isolates were random mutants rather than true transformants, or that the macrolide resistance observed in Ci115 may be multifactorial and that all contributors to the resistance were not transferred to any transformant. Multiple attempts at transformation using genomic DNA from M3, each involving changes in conditions such as the concentration of selective agent used in the growth media and the volume of DNA used in the reaction, were not successful. This may be because the specific alterations in M3 that are contributing to macrolide resistance are simply unable to be transformed into our recipients using our methodology, or because the alterations are incapable of increasing macrolide resistance on their own and would need to be transformed in unison to see the effect (the odds of transferring everything that is contributing to macrolide resistance is low). We used multilocus sequence typing to characterise the donors and recipients by extracting the loci *adk*, *atpG*, *frdB*, *mdh*, *pgi* and *recA*, as previously described,[283] from the spreadsheets previously generated for these organisms by RAST. This was used to generate a phylogenetic bootstrap cladogram (see Figure 5.2) in the Molecular Evolutionary Genetics Analysis (MEGA) software package[284] with the donors and recipients along with commercially available strains of known identity. M3 appeared to be more phylogenetically distinct from the recipients than Ci115 was and this may have also contributed to the inability for our recipients to be successfully transformed with genomic DNA from M3, although there is little precedent for phylogenetic relationships affecting transformation efficiency and it would be necessary to attempt transformation with genomic DNA of isolates that are phylogenetically closely related to M3 to further investigate this possibility.

For CA1 (AZ MIC = 64 μg/mL) and CA3 (AZ MIC = 32 μg/mL), the isolates derived from

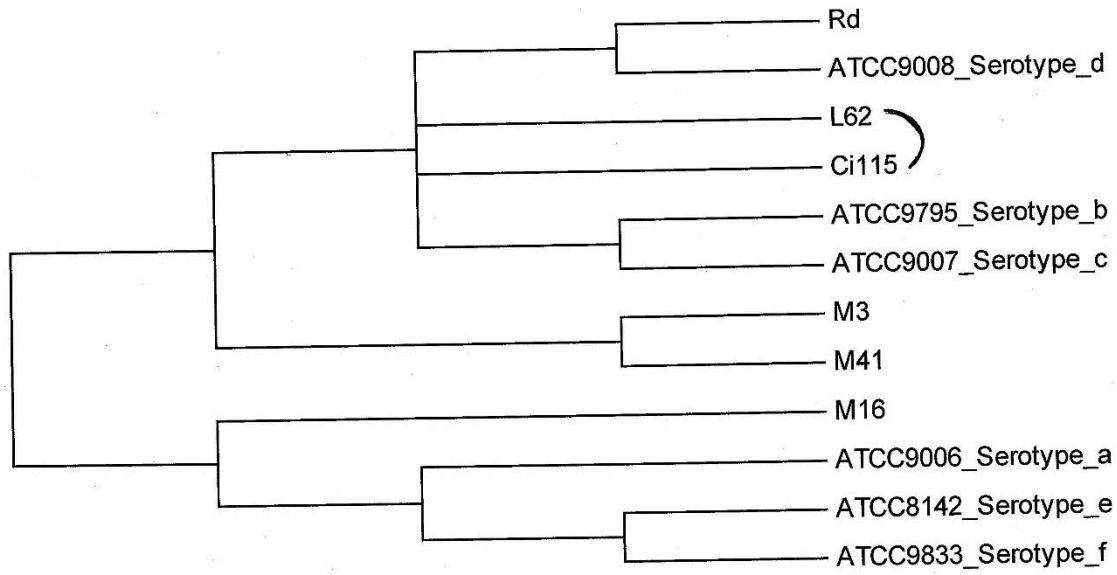

Figure 5.2: Phylogenetic cladogram of donors and recipients alongside commercial strains. M3 appeared to be genetically distinct from Ci115 and the recipients. Note that L62 represents 86-028NP in this figure.

experiments using Ci115 as the donor and NTHi 86-028NP as the recipient, we observed that neither isolate received any components of 23S from Ci115 (see Figure 5.3). CA1 did not have any changes in L4 or L22, but appeared to receive a 36kb-sized segment of DNA from Ci115 (see Figure 5.4). This transformation generated a large number of single nucleotide polymorphisms (snps) in CA1 across multiple genes, including a cluster of *fts* genes (see Table A2.1 in Appendix 2). It is not immediately clear how these snps would affect macrolide resistance as they have not occurred in regions known to affect macrolide susceptibility. While determining the exact role of these snps is beyond the scope of this study, it does raise questions as to how or if this particular segment of DNA is contributing to the high macrolide resistance phenotype in Ci115, especially in relation to the effect of the previously observed C2611U alteration in 23S and the R88P alteration in L22 of Ci115.

CA3 carried a DEL 299CTA in the L22 gene that resulted in an in-frame deletion of threonine at position 100 of the L22 protein. CA3 was also observed to carry a snp in *nth*, an endonuclease coding gene, which was also seen in CA1 (see Table A2.1 in Appendix 2), although this is unlikely to be contributing to the macrolide resistance seen in CA3. No other changes were observed compared to the reference, indicating that the L22 alteration is the most likely cause of the macrolide resistance in CA3. However, as the specific change observed in CA3 was not seen in Ci115, it is most likely not a transferred deletion but rather a random mutational change in this specific transformant.

Neither CA4 (AZ MIC = 96 µg/mL) nor CA6 (AZ MIC = 24 µg/mL), the isolates derived from experiments using Ci115 as the donor and *H. influenzae* Rd KW20 as the recipient, carried any alterations in 23S. Both transformants appeared to exhibit a large degree of sequence variation compared to the recipient strain and the vast majority of snps were detected in both transformants (see Tables A2.2a-d in Appendix 2 for complete list of

Figure 5.3: Comparison of 23S of CA1, CA3 and NTHi 86-028NP, each compared to a reference 23S sequence. Each sequence carried the same snps compared to the reference, indicating that there was no variation between the sequences and that no components of Ci115 were transferred to the recipient.

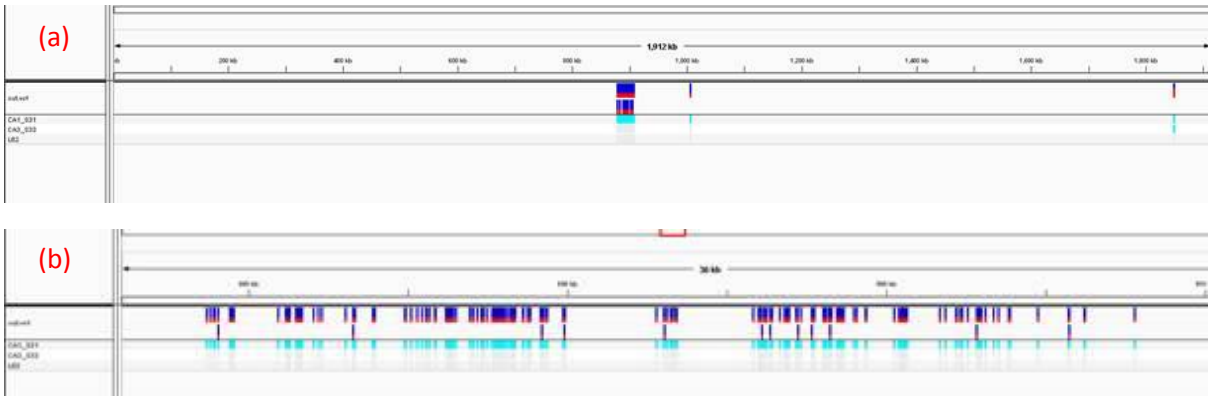

Figure 5.4: Snp density plot of CA1, CA3 and NTHi 86-028NP (a). CA1 received a 36kb-sized segment of DNA from Ci115 that CA3 did not receive. A close-up view of this sequence (b) demonstrates numerous snps compared to the recipient.

alterations), including the macrolide resistance-associated R88P alterations in L22 originally detected in Ci115 (corresponding nucleotide alteration is highlighted in yellow in Table A2.2b). This finding indicates that the presence of R88P alone is not necessarily sufficient to cause the high-level macrolide resistance observed in the donor, although it may still be one of several contributors. The snps observed in CA4 and CA6 are distinct from those observed in CA1. A number of identical insertions and deletions in both transformants were also apparent (see Tables A2.3a-b in Appendix 2). CA4 appeared to recombine to a greater extent than CA6, with CA4 bearing 3 additional clusters of snps that CA6 lacked (see Tables A2.4a-c in Appendix 2), located within the same 1.1 Mbp region (see Figure 5.5), as well as 4 additions insertions or deletions (see Table A2.5 in Appendix 2). These additional recombination events in CA4 are interesting to note with consideration of the fact that CA4 had a higher azithromycin MIC than CA6, although it is not clear how these additional components that CA4 carried affect macrolide resistance.

Overall, these transformation studies have failed to demonstrate any single transferable genetic feature to account for the high azithromycin MIC in Ci115 or M3. The only slightly raised azithromycin MICs in the transformants are primarily of academic interest and cannot be easily explained by the findings reported here, although the findings hint at a number of novel potential hotspots with involvement in macrolide resistance. While further investigating this possibility is beyond the scope of the present study, future studies attempting to identify unknown markers of resistance in Ci115 could utilise the very recently described technique of Transformed Recombinant Enrichment Profiling (TREP), which would involve using genomic DNA from Ci115 to generate a large pool of recombinants of a suitable recipient (such as *H. influenzae* Rd KW20), enriching those recombinants with increased resistance to macrolides, and then deep sequencing the enriched pool of recombinants to identify potential loci that are contributing to this resistance. This has

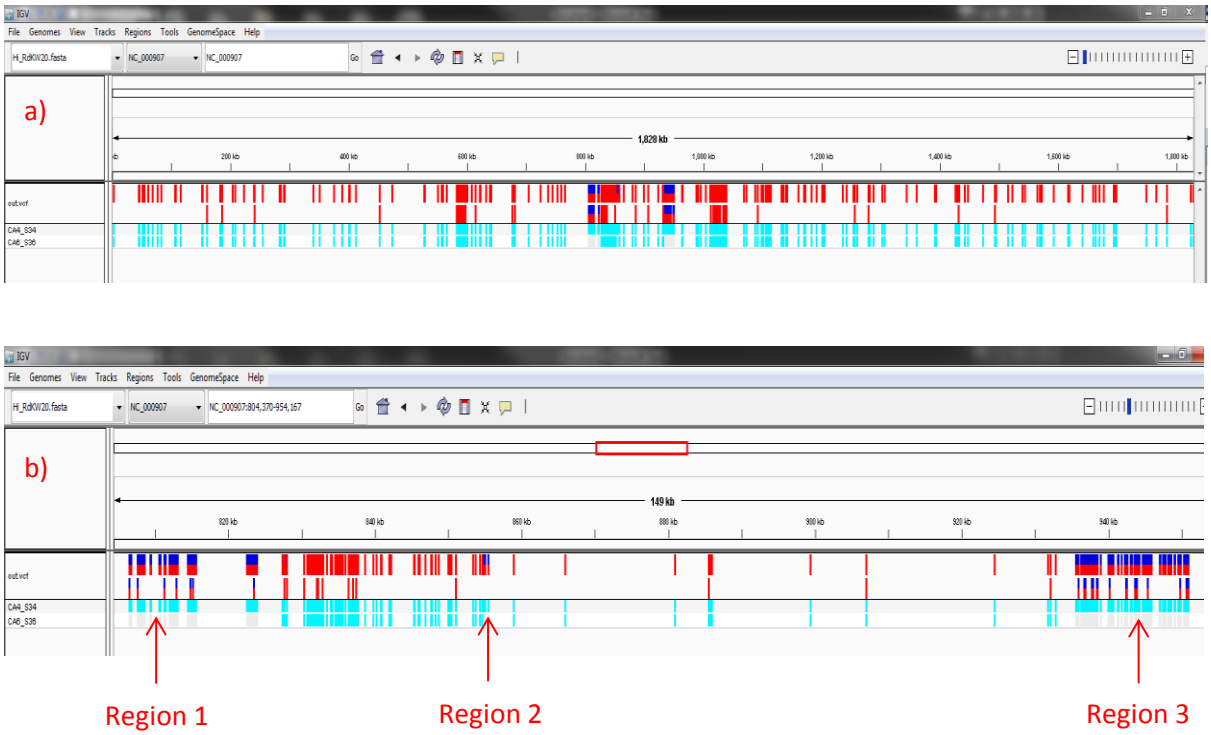

Figure 5.5:Snp density plot of CA4 and CA6 (a). A close-up view of this sequence (b) reveals that CA4 received three additional segments of DNA from Ci115 that CA6 did not receive.

previously been used to identify loci responsible for invasion in *H. influenzae*[285] and could potentially be applied here. In any case, the fact that the C2611U alteration of 23S observed in Ci115 was not transferred to any transformants and those transformants subsequently exhibited MICs that were lower than Ci115 further supports the finding of multi-copy 23S mutations being the primary mechanism of resistance in Ci115. However, the findings reported here may be indicative of a multifactorial aetiology for the high-level macrolide resistance in Ci115, with resistance resulting from an accumulation of factors of which only some were demonstrated to be transferable.

The BLAST score output given by the SPANDx pipeline used to screen for the presence of select AMRGs in the NTHi WGSs (n=92) included in this study is summarised in Tables A2.6a-g, A2.7a-g and A2.8a-g in Appendix 2. For each gene included in this study, these scores were ≤0.21 for all WGSs, indicating that these genes were not present in this particular selection of isolates. The BLASTn analysis performed on Ci115 and M3 in CARD also failed to detect any known AMRGs in these isolates. When the fasta files for these isolates were submitted to RGI in CARD, the output for both analyse produced a single match that was annotated as a "macrolide resistance gene". The match given was for CRP (see Figure 5.6 for output), an inherent catabolite regulatory protein that has previously been implicated as a regulator of the MdtEF multi-drug efflux pump in *E. coli*.[286] Deletion of *crp* in *E. coli* has previously been reported to increase resistance to erythromycin and azithromycin,[286] but CRP appears to be unaltered in Ci115 and M3 compared to NTHi 86-028NP (see Figure 5.7) and is unlikely to be contributing to the high-level macrolide resistance observed here. In any case, no known AMRGs were detected in Ci115 and M3 using RGI either. Our inability to detect these genes using the SPANDx system or CARD further supports the idea that the macrolide resistance observed in these isolates is not due to the acquisition of AMRGs but to the presence of alterations in 23S and other chromosomal components. Furthermore, the fact

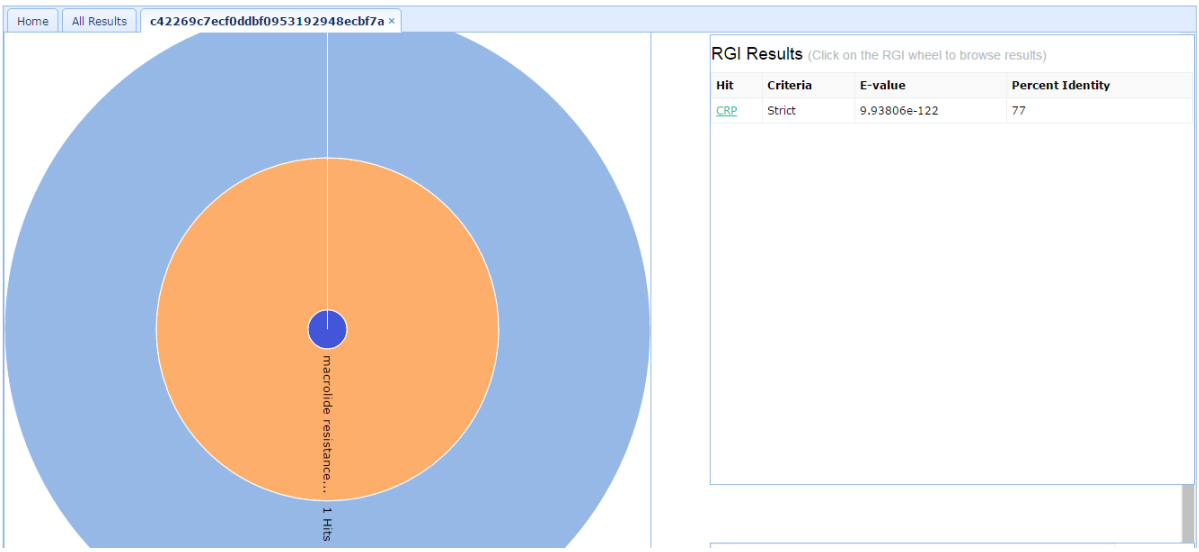

Figure 5.6: RGI output for Ci115 and M3.

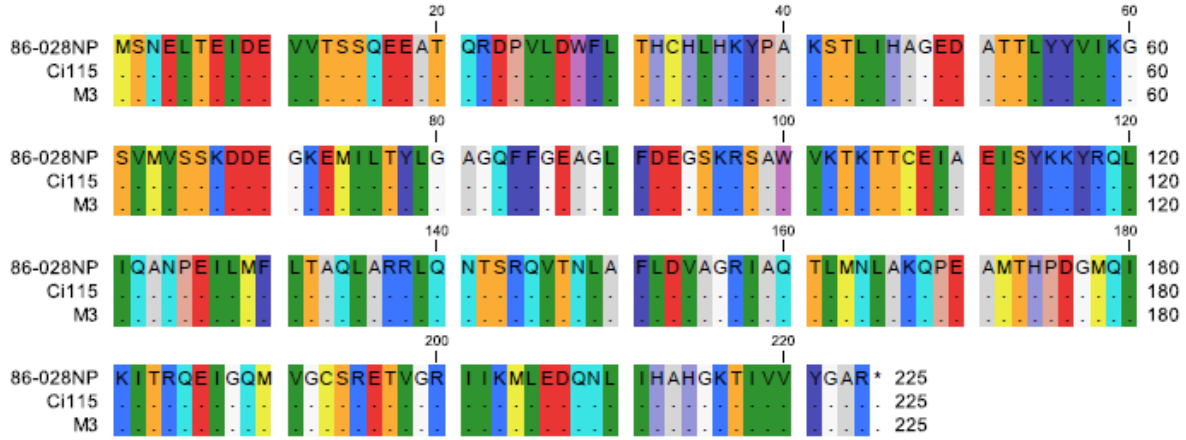

Figure 5.7: Sequences of CRP of Ci115 and M3 aligned against NTHi 86-028NP.

that these genes (including the 6 genes investigated in Chapter 4) were not detected among any of the other isolates included in this study further indicates that AMRGs are currently not widespread among NTHi isolates.

It is important to note that the genomes included in this study were not necessarily closed genomes. The genomes were assembled from reads of varying size, but there was insufficient overlap present across these reads to generate a closed genome. This raises a number of issues when attempting to analyse these genomes for the presence of resistance markers. Firstly, this can result in gaps in the assembled sequences that may have caused important features to be missed during the analysis. In addition, this makes it difficult to handle segments for which there are multiple copies, such as 23S rRNA, which were assembled together as one segment. In this instance, dosage of particular mutations could still be estimated through the extrapolation of the output in IGV stating the percentage of reads carrying the alteration, but it was not possible to state which copy of 23S carried a particular mutation in situations where dosage was not complete.

It is important to stress that the work described here represents preliminary studies in regards to how whole genome sequencing could be utilised in the investigation of macrolide resistance in NTHi. While the findings of this study regarding chromosomal alterations do raise some interesting questions regarding macrolide resistance mechanisms, further work would be required to establish the exact role the numerous alterations observed within the transformants generated in this study have in causing resistance to macrolides in NTHi, as well as to establish the importance of 23S rRNA dosage in *H. influenzae*. However, based on the whole genome sequencing and analyses we have completed in this chapter, we conclude once again that resistance to macrolides in NTHi can primarily be attributed to alterations in specific chromosomal components rather than through the acquisition of macrolide resistance genes, and that AMRGs are not currently widespread among NTHi isolates.

Chapter 6: Phenotypic effect of expression of select acquired macrolide resistance genes in *Haemophilus influenzae*

6.1 Introduction

Most isolates of *H. influenzae* exhibit low-level intrinsic non-susceptibility to macrolide antibiotics, attributable to the presence of an inherent efflux mechanism analogous to the acrAB system in *E. coli*. Despite this, there is increasing interest in utilising macrolides for respiratory infections where NTHi may be involved.[25,29] While the efficacy of these agents in treating infections with wild-type NTHi has already been questioned in some instances,[29,274] the potential for NTHi to acquire high-level macrolide resistance in response to macrolide therapy may further undermine the utility of these agents.

High-level macrolide resistance in NTHi has historically been attributed to chromosomal alterations of the bacterial ribosome structure that inhibit macrolide binding, including alterations in 23S rRNA, and the L4 and L22 ribosomal proteins. In contrast, acquired macrolide resistance genes (AMRGs) have not been widely reported among NTHi isolates and a number of studies investigating macrolide resistance in *H. influenzae* have failed to detect these genes.[29,251-253] In the single study where AMRGs have been reported in NTHi, the effect of these genes on phenotypic resistance was inconsistent.[93] In that study, among a collection of 106 NTHi isolates from children with cystic fibrosis enrolled in a placebo-controlled azithromycin trial, all isolates had at least one of the AMRGs *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A). However, only 27 of these were phenotypically resistant to macrolides, with the remaining isolates either intermediate (n=78) or susceptible (n=1). In addition, some of these NTHi isolates were able to conjugatively transfer some or all of their

AMRGs to *H. influenzae* Rd KW20 or *E. faecalis* JH2-2 recipients with an associated moderate to large increase in erythromycin and azithromycin MICs.[93]

While the findings of this study are remarkable and may suggest a possible role for AMRGs in producing a macrolide resistance phenotype in NTHi, the variable macrolide resistance phenotype observed in the AMRG-carrying NTHi isolates reported in that study raises questions about the ability of these genes to confer high-level macrolide resistance in NTHi. Therefore, the aim of this study was to investigate and clarify the expression and phenotypic effect of select AMRGs in *H. influenzae*. A range of approaches were taken to generate AMRG-positive *H. influenzae*, including conjugative transfer from wild-type Gram positive donors, and cloning with and without associated regulatory regions.

6.2 Methods and Materials

6.2.1 Bacterial strains

The complete list of donor isolates used in this study can be found in Table 6.1. All were Gram positive organisms of human origin that were defined as erythromycin resistant using EUCAST methodology and interpretive criteria (for staphylococci, MIC > 2 µg/mL; for streptococci, MIC > 0.5 µg/mL) and that carried at least one of *erm*(A), *erm*(B), *erm*(C), *mef*(A) and *mef*(E). Locked nucleic acid dual-labelled hydrolysis probes (Sigma-Aldrich, NSW, Australia) were used to detect the presence of these genes in each of the donor isolates as previously described in Chapter 4 of this thesis. All seven donor isolates were used for conjugation assays, while isolates U37 (*erm*(A)), U200 (*erm*(B)), U387 (*erm*(C)), A22 (*mef*(A)) and A7 (*mef*(E)) were additionally selected for use in cloning studies. D tests for the detection of inducible clindamycin resistance were performed on all *erm*–carrying cloning study strains as previously described[287] (see Table 6.1).

For conjugation assays, *H. influenzae* Rd KW20 was used as the recipient. Both *H. influenzae* Rd KW20 and *E. coli* JM109 were used during the cloning assays (see below for further details).

6.2.2 Conjugation of AMRGs into *Haemophilus influenzae* Rd KW20

To confirm that our selected *H. influenzae* Rd KW20 strain was receptive, mating experiments were performed to transfer ICE*Hin1056* from a previously generated *H. influenzae* Rd KW20 transconjugant (donor) to a separate streptomycin-resistant *H. influenzae* Rd KW20 mutant (recipient). The donor and recipient were mixed in a ratio of 1:5 and transconjugants were grown on chocolate agar supplemented with streptomycin (500 µg/mL) and ampicillin (4 µg/mL).

Table 6.1: Donor sources of AMRGs used in this study.

Isolate ID	Species	Gene of Interest	D test	AZ MIC (µg/mL)	ERY MIC (µg/mL)
U37*	MRSA	*erm*(A)	+	≥64	≥64
A20	*S. pneumoniae*	*erm*(B)	ND	ND	≥1
U200*	*S. pneumoniae*	*erm*(B)/*mef*(E)	-	≥64	≥64
U387*	*S. aureus*	*erm*(C)	+	≥64	≥64
A22*	*S. pyogenes*	*mef*(A)	ND	32	4
A7	Group G *Streptococcus*	*mef*(E)	ND	≥64	16
A10*	*S. pneumoniae*	*mef*(E)	ND	ND	≥1

AZ, azithromycin; ERY, erythromycin; ND, not determined

*Source of genes for cloning studies. Note that U200 carries both *erm*(B) and *mef*(E), but only *erm*(B) was cloned from this isolate.

To transfer each of the AMRGs of interest to *H. influenzae* Rd KW20, mating experiments were performed using the Gram positive isolates listed in Table 6.1 as donors and the streptomycin-resistant *H. influenzae* Rd KW20 mutant as the recipient. The donor and recipient were mixed in ratios of 1:1, 1:5 and 1:10; transconjugants were grown on chocolate agar with streptomycin (500 µg/mL) and erythromycin (8 µg/mL and 16 µg/mL). Refer to Chapter 3 for further information.

6.2.3 Cloning and transformation of AMRGs into *Haemophilus influenzae* Rd KW20

Cloning into *H. influenzae* is complicated by the absence of suitable vectors, as most commercial vectors are designed for use in *E. coli* and do not replicate in *H. influenzae*. This laboratory has frequently used the shuttle vector pLS88 and we have previously observed significant run-off transcription of open reading frames (ribosome binding site present but promoters absent) cloned into the *Eco*RI restriction site. We sought to confirm this and determine the importance of cloning orientation by cloning bla_{TEM-1} (where effect of expression on phenotype is known) as a prelude to cloning the acquired macrolide resistance genes.

6.2.3.1 Cloning of bla_{TEM-1} and AMRGs into *Escherichia coli* JM109

The kanamycin resistance-marked plasmid pLS88 was extracted from a previously generated pLS88-carrying *E. coli* in accordance with the protocol outlined in the QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany). The plasmid was digested with *Eco*RI (Promega) at 37°C for 3 hours, followed by an incubation of 65°C for 15 minutes to deactivate the enzyme. Subsequently, the plasmid was digested with alkaline phosphatase (*AP*) (Promega, calf intestinal) at 37°C for 3 hours to prevent re-ligation.

To prepare the *bla*$_{TEM-1}$ insert for cloning, pGEM-3Z (Promega) was used as a template for PCR using *Eco*RI-tailed *bla*$_{TEM-1}$ primers as listed in Table 6.2. To prepare the AMRGs for cloning into pLS88, genomic DNA was extracted from each of the selected donor isolates. Using the genomic DNA as a template, the inserts were generated by PCR using the *Eco*RI-tailed gene specific primers listed in Table 6.2. In all instances, primers were designed such that the amplicon included the RBS. Sequencing was performed to confirm the identity of the AMRG inserts, using the primers listed in Table 6.3. The AMRGs and *bla*$_{TEM-1}$ were digested with *Eco*RI as above.

To construct the recombinant plasmids, the digested amplicons were ligated with the linearised phosphatase-treated plasmid using T4 DNA ligase (Promega). Maps of these vectors (excluding *bla*$_{TEM-1}$) are shown in Appendix 3 of this thesis. These constructed plasmids were combined with commercially prepared *E. coli* JM109 recipient cells (Promega) and incubated on ice for 30 minutes. The cell-plasmid mixture was then heat-shocked at 42°C for 20 seconds before being returned to ice for 2 minutes. The heat-shocked cells were suspended in 450 µL SOC media (Bioline) and grown at 37°C for 80 minutes before being plated on LB media supplemented with kanamycin (30 µg/mL).

The presence of respective AMRGs in the resulting *E. coli* clones were confirmed using locked nucleic acid dual-labelled hydrolysis probes as previously described in Chapter 4 and the presence of *bla*$_{TEM-1}$ confirmed by PCR (*bla*$_{TEM}$-F, 5'-GAAGACGAAAGGGCCTCGTG-3'; *bla*$_{TEM}$-R, 5'-GGTCTGACAGTTACCAATGC-3') as previously described.[288] The inserts in the clones were amplified using primers designed for the amplification of regions flanking the insertion site of pLS88 (pLS88seq-F, 5'-GACTTCATCCGCACACAC-3'; pLS88seq-R, 5'-CAGGCAACCAGTCAGAAT-3'); amplicons were submitted to the Australian Genome Research Facility for sequencing (as previously described in Chapter 3 of this thesis) to confirm that the cloned genes were complete.

Table 6.2: Primers used for the preparation of bla_{TEM-1} and AMRG inserts into pLS88.

Gene/Region*	With promoter?	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence (5' – 3')
bla_{TEM-1}	No	Preparation of bla_{TEM-1} insert	55	TEM-CL-F	GCGCGAATTCAAAAAGGAAGAGTATG
				TEM-CL-R	GCGCGAATTCACCTAGATCCTT
erm(A)	No	Preparation of *erm*(A) insert		ermA-CLNG-F	GCGCGAATTCTGAACGCTTAATGTCAGTTCATT
				ermA-CLNG-R	GCGCGAATTCGCGTCCTCTTGTGAAATTAGAGA
erm(A)	Yes	Preparation of *erm*(A) insert	57	ermA-clprF	GCGCGAATTCATCGTAATCTTCTGCAACCT
				ermA-clprR	GCGCGAATTCAGCGTCCTCTTGTGAAAT
erm(B)	No	Preparation of *erm*(B) insert	55	ermB-CLNG-F	GCGCGAATTCTTTATAGATGTAATCACTTCAGGA
				ermB-CLNG-R	GCGCGAATTCCCAAATTTACAAAAGCGACTCA
erm(B)	Yes	Preparation of *erm*(B) insert	51	ermB-clprF	GCGCGAATTCAGAGTGTGTTGATAGTG
				ermB-clprR	GCGCGAATTCGAATTATTTCCTCCCGT
erm(C)	No	Preparation of *erm*(C) insert	55	ermC-CLNG-F	GCGCGAATTCCAAATTAAAGAGGGTTATAATGAACG
				ermC-CLNG-R	GCGCGAATTCTGCAGTTTATGCATCCCTTA
erm(C)	Yes	Preparation of *erm*(C) insert	54	ermC-clprF	GCGCGAATTCGCTCTACGACCAAAACTA
				ermC-clprR	GCGCGAATTCGCAGTTTATGCATCCCTT
mef(A)/*mef*(E)	No	Preparation of *mef*(A)/*mef*(E) insert	55	mef-CLNG-F	GCGCGAATTCTCAAGCAAAAATAATATGCAGGAGA
				mef-CLNG-R	GCGCGAATTCCAGGAAGAGTTACATGAAAATAAGA
mef(A)/*mef*(E)	Yes	Preparation of *mef*(A)/*mef*(E) insert	54	mefE-clprF	GCGCGAATTCCAGACCAAAAGCCACATT
				mefE-clprR	GCGCGAATTCGCGATTTTAGCAGGAAGA

*The *mef* primers were used for *mef*(A) and *mef*(E).

Table 6.3: Primers used to sequence inserts after preparation.

Primer	Sequence (5' – 3')
ermA-106f[289]	GAA ATY GGR TCA GGA AAA GG
ermA-437r[289]	AAY AGY AAA CCY AAA GCT C
ermB-91f[289]	GAT ACC GTT TAC GAA ATT GG
ermB-454r[289]	GAA TCG AGA CTT GAG TGT GC
ermC-43f[289]	TCA AAA CAT AAT ATA GAT AAA
ermC-684r[289]	GCT AAT ATT GTT TAA ATC GTC AAT
mefA-MF6[290]	GGA CCT GCC ATT GGT GTG
mefA-MF4[290]	ACC GAT TCT ATC AGC AAA G

6.2.3.2 Electroporation of *Haemophilus influenzae* recipient cells

H. influenzae Rd KW20 cells were grown to an optical density at 650 nm (OD_{650}) of 0.5 in Heart Infusion Broth (Oxoid) supplemented with Vitox (Oxoid) and 15 µg/mL NAD and haematin (Oxoid) at 37°C on a shaker.

Cells were subsequently chilled for 30 minutes and washed 5 times with PSG buffer (15% glycerol, 272 mM sucrose, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4) at 4°C. Following washes, cells were resuspended in 1 mL cold PSG buffer. The plasmids carrying the AMRGs or bla_{TEM-1} were introduced into the washed cells by electroporation. Cells were electroporated in 2 mm cuvettes using the Bio-Rad MicroPulser™ set on the EC2 setting of the MicroPulser (voltage of 2.5kV and time constant of approximately 5 ms). Electroporated cells were suspended in sBHI and incubated at 37°C on a shaker for 4 hours. Transformants were plated on chocolate agar supplemented with kanamycin (30 µg/mL) and grown overnight in 37°C in CO_2. Transformants were confirmed using locked nucleic acid dual-labelled hydrolysis probes as previously described in Chapter 4.

6.2.3.3 Determination of insert orientation

Primers for the amplification of regions flanking the insertion site (listed in section 6.2.3.1) were designed to determine and subsequently confirm the inserts were in the correct orientation for expression. To determine which orientation was "correct", select bla_{TEM-1}-carrying *H. influenzae* transformants first underwent ampicillin disc diffusion susceptibility testing in accordance with the recommendations of EUCAST[291] alongside an untransformed control to create 2 groups of transformants: one group of resistant transformants and one group of susceptible transformants. To determine the insert direction of these groups of transformants, 2 separate PCR reactions were performed on each transformant. One reaction used pLS88seq-F and TEM-CL-F, and the other reaction used pLS88seq-F and TEM-CL-R;

our rationale was that only one of these reactions should result in an amplicon depending on the direction. These PCRs revealed that resistant transformants produced an amplicon with the reaction involving TEM-CL-R only, while the susceptible transformants produced an amplicon with the reaction involving TEM-CL-F only.

These results were used to develop a screening method to the determination of the orientation of the inserts in the AMRG-carrying transformants (without natural promoters only). This screen involved testing select transformants as above, but substituting the TEM primers for the corresponding AMRG primers listed in Table 6.3. Transformants that produced an amplicon with the corresponding reverse primers only were deemed to carry the insert in the correct orientation for expression.

6.2.4 Macrolide susceptibility testing and D tests

Erythromycin and azithromycin MICs for the transformants were determined by broth microdilution as previously described in Chapter 3. D tests were performed on all *erm* "native promoter" transformants as previously described.[287]

6.2.5 Expression analysis

To prepare transformants for RNA extractions, isolates were grown overnight on chocolate agar supplemented with kanamycin (30 µg/mL) to maintain the plasmid. The overnight growth was subcultured to 10 mL of sBHI supplemented with kanamycin (30 µg/mL) and incubated in air at 37°C on a shaker (180 rpm) until reaching early- to mid-log phase growth (represented by an absorbance of approximately 0.5 at 650 nm). In addition, for those transformants with AMRGs cloned with natural promoters, broths were prepared as above but with the addition of erythromycin at a level of 0.125 of the previously determined MIC for each transformant.

After reaching log-phase growth, cells were chilled on ice for 30 minutes before RNA extractions were performed on all AMRG-carrying *H. influenzae* transformants as previously described in Chapter 3. DNA was quantified using the locked nucleic acid dual-labelled hydrolysis probes (Sigma-Aldrich, NSW, Australia) previously described in Chapter 4 of this thesis. *gyrA* was selected as the housekeeping gene for all transformants (F primer, 5'-TCCAATCATCTATCACCC-3'; R primer, 5'-TTACATCACCCACAACAC-3'; probe, 5'-TGCACGCCCAACGATAACC-3'). All samples were run in parallel with a no-RT control to ensure no contamination with genomic DNA was present. ΔCT values for each transformant were calculated using the formula $\Delta CT = 2^{-(CT(GOI)-CT(gyrA))}$.

Comparisons of ΔCT values were performed using unpaired t-tests. Firstly, ΔCT values produced by each of the 5 AMRG-carrying transformants (with no natural promoter and no erythromycin exposure) were compared to ΔCT of untransformed *H. influenzae* Rd KW20 to determine if any increase in the mRNA change was statistically significant. Comparisons were also performed between each of the 5 AMRG-carrying transformants to determine if the amount of expression was consistent across all transformants. Secondly, for each of the five AMRGs, ΔCT comparisons were performed between transformants with their own natural promoters and transformants without their own natural promoters. Finally, for each transformant with their natural promoters, ΔCT comparisons were performed between transformants grown in broth without erythromycin exposure and transformants grown with erythromycin exposure. A p-value of 0.05 was used as a cut-off for significance for all tests.

6.3 Results and Discussion

The aim of this study was to investigate the effect of expression of select acquired macrolide resistance genes in *H. influenzae*. Initially, we attempted to naturally transfer these genes to *H. influenzae* Rd KW20 by conjugation. We were able to efficiently transfer ICE*Hin1056* into our designated recipient by conjugation, demonstrating that the strain was receptive and that our conjugation protocol was appropriate. However, our attempts at transferring AMRGs from the Gram positive donors were not successful in generating transconjugants using the method described above.

This is in contrast to the Roberts et al. (2011) study, in which these genes were successfully transferred from NTHi donors to both *H. influenzae* and *E. faecalis* recipients with an associated increase in macrolide MICs. In reviewing the findings of Roberts et al., we noted a number of issues with the way in which transconjugants were detected and analysed. While Roberts et al. were able to confirm the presence of the various AMRGs of interest in the transconjugants generated in that study, none of the transconjugants were analysed for the presence of macrolide resistance-associated L4 or L22 mutations, or 23S rRNA alterations. In the work described in Chapter 5 of this thesis, we were able to generate spontaneous macrolide resistant mutants of *H. influenzae* Rd KW20 by selection on media supplemented with macrolides (azithromycin, 16 µg/mL); this resistance was associated with a DEL 299CTA in the L22 gene. In addition, the range of conjugation efficiencies reported in the Roberts study ($2.5x10^{-10}$ transconjugants/recipient – $3.7x10^{-7}$ transconjugants/recipient) are not drastically different from previously reported macrolide resistance-associated L4, L22 and 23S rRNA mutation frequencies in *H. influenzae* mutants placed under selective pressure with inhibitory levels of azithromycin and clarithromycin.[273] Given that the azithromycin MIC values observed in such mutants have been shown to be at around the same level as that observed in the transconjugants generated in the Roberts study (depending on the mutation

type),[93,273] we feel that it may have been valuable for Roberts et al. to analyse all transconjugants for the presence of L4, L22 and 23S rRNA alterations to determine that these were not contributing to the raised MICs observed in the transconjugants and further support the conclusion that the AMRGs could act as a resistance determinant in NTHi.

In designing our conjugation experiments, we were not certain of how great an extent the erythromycin MIC would change in *H. influenzae* as a result of acquiring these genes, and as such it was difficult to determine the appropriate erythromycin concentration in our selective plates. Roberts et al. used a concentration of 5 µg/mL for the selection of *H. influenzae* transconjugants,[93] but we found that our particular *H. influenzae* Rd KW20 recipient strain was not inhibited at this concentration and therefore used concentrations of 8 and 16 µg/mL instead. Given that some of the transconjugants obtained in the Roberts et al. study had erythromycin MICs as low as 12 µg/mL,[93] it is possible that 8 µg/mL may have been too high to allow for transconjugants to grow effectively on our media. For future studies, it may be more appropriate to select a different recipient strain that is more easily inhibited at lower concentrations of erythromycin to increase the chance of recovering transconjugants.

It is worth noting that the range of AMRG donor isolates we had access to and the range of donors used by Roberts et al. represent a key difference between the two studies. When attempting to conjugatively transfer AMRGs to *H. influenzae* Rd KW20, we were unable to attempt to replicate the works of Roberts et al. (2011) because we did not have access to any wild-type NTHi isolates with AMRGs. We instead used select AMRG-carrying Gram positive organisms such as *S. aureus* and various streptococci as donors for these experiments. The use of these species as donors was deemed appropriate in this case because these species are both known to be involved in respiratory infections and common carriers of the particular AMRGs included in this study. These species can additionally coexist with NTHi within the same respiratory niche, creating a significant opportunity for the natural

transfer of these AMRGs to NTHi; indeed, Roberts et al. put forth the possibility of the occurrence of AMRG transfer between species coexisting in the lungs or oral cavity[93], and Luna et al. had previously been able to demonstrate conjugative transfer of *mef* from *S. pneumoniae* to *H. influenzae*.[255] We therefore felt that demonstrating AMRG transfer from these Gram positive donors to *H. influenzae* would be especially relevant in assessing the potential for these genes to emerge as threats in NTHi. Ultimately we were unable to demonstrate conjugative transfer using these donors. It is worth noting that the main precedent for this natural transfer of AMRGs from Gram positive respiratory organisms to NTHi is the Roberts study, and there is otherwise little experimental evidence available for such transfer given that these genes are not widely seen in NTHi despite the species sharing a niche with Gram positive AMRG carriers. Given that this transfer would need to cross a significant taxonomic barrier, it may simply be that these genes very rarely transfer to NTHi despite the aforementioned opportunities in which it could occur, and that it may only occur under very specific circumstances involving selective pressure from macrolide maintenance therapy observed in the Roberts study.

As we were not able to generate AMRG-carrying *H. influenzae* transconjugants, we instead decided to clone the genes into a shuttle vector and use this construct to transform *H. influenzae* Rd KW20. Out initial requirement was to observe the macrolide resistance effect of the expression of AMRGs in *H. influenzae,* and given the complexity of the regulatory regions of many AMRGs, our first option was to clone the AMRGs without native promoters such that they would be expressed from run-off transcription from the plasmid itself. The calculated ΔCT values for each of these "no native promoter" transformants were statistically significantly increased compared to that of the untransformed *H. influenzae* Rd KW20 control (*erm*(A), p=0.0047; *erm*(B), p=0.0031; *erm*(C), p<0.0001; *mef*(A), p=0.0037; *mef*(E), p=0.0018; see Figure 6.1), for which no amplification occurred using the AMRG locked

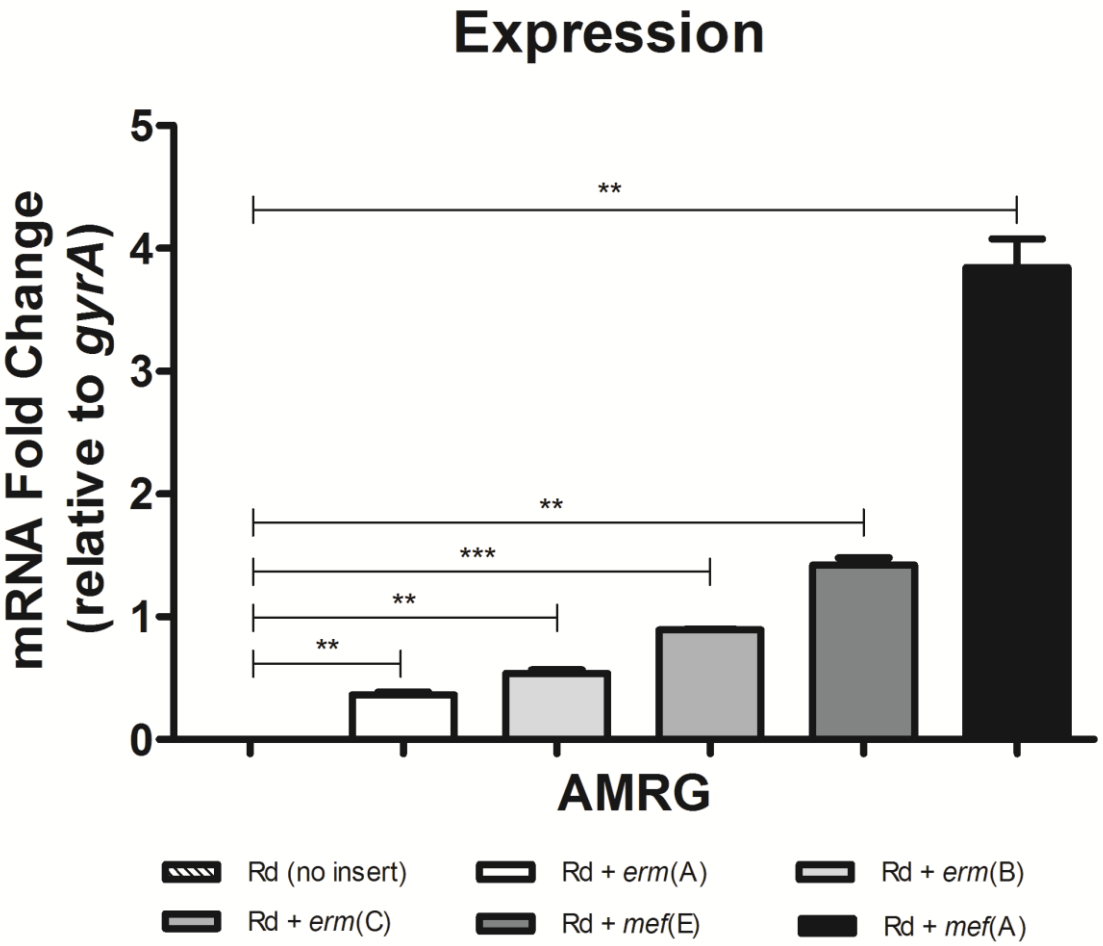

Figure 6.1: ΔCT values (mRNA Fold Change (relative to *gyr*A)) for each of the 5 transformants ("no native promoter") and untransformed *H. influenzae* Rd KW20. Note that each transformant only amplified with the primer set corresponding to the gene of interest; the columns represent expression of the gene of interest for each transformant only. Untransformed *H. influenzae* Rd KW20 produced no amplification with any primer set. Expression of each AMRG was statistically significantly increased compared to the untransformed *H. influenzae* Rd KW20.

*, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

nucleic acid dual-labelled hydrolysis probes, indicating that all genes were being expressed in *H. influenzae*. Interestingly, when the ΔCT values of each transformant were compared to one another, the ΔCT values differed significantly (see Table 6.4 and Figure 6.2), indicating that the level of expression of each gene was not constant across the transformants despite being cloned into the same site on the same plasmid. These results were confirmed by a repeat experiment, using the same transformants but fresh RNA isolation, cDNA generation and quantification, and are not easily explained.

The MIC results for the "no native promoter" transformants are listed in Table 6.5. Of the *erm* genes included in this study, the *erm*(A) transformant (erythromycin MIC = 16 µg/mL, azithromycin MIC = 4 µg/mL) did not appear to have a large increase in MIC compared to the untransformed *H. influenzae* Rd KW20 strain (erythromycin MIC = 8, azithromycin MIC = 4). By comparison, the *erm*(B) (erythromycin MIC = 64 µg/mL, azithromycin MIC = 32 µg/mL) and *erm*(C) (erythromycin MIC > 64 µg/mL, azithromycin MIC > 64 µg/mL) transformants had much larger MIC increases. This is reflected by the differences in ΔCT values for these transformants, with the *erm*(A) transformant having significantly lower expression than that of the other *erm* transformants. By contrast, the MIC values for the *mef*(A) and *mef*(E) were unchanged from the untransformed *H. influenzae* Rd KW20 strain. This is despite the expression analyses demonstrating expression of these genes was even higher than that of the *erm* genes. These observations may reflect different efficiencies of the respective enzyme products of the genes.

In the Roberts study (in which *mef*(A) and *mef*(E) were not differentiated), *mef*(A) was successfully transferred by conjugation and resulted in the generation of transconjugants exhibiting moderate erythromycin and azithromycin MIC increases in the order of 2- to 24 fold and 5- to 15-fold, respectively.[93] It is therefore interesting to note that our *mef*(A) and *mef*(E) transformants did not exhibit any increase in MICs of these antibiotics. As previously

Table 6.4: Transconjugant pair ΔCT comparisons.

	p*-values				
Vs.	**Rd KW20 *erm*(A)**	**Rd KW20 *erm*(B)**	**Rd KW20 *erm*(C)**	**Rd KW20 *mef*(A)**	**Rd KW20 *mef*(E)**
Rd KW20 *erm*(A)		0.0464	0.0023	0.0046	0.0038
Rd KW20 *erm*(B)			0.0073	0.0051	0.0058
Rd KW20 *erm*(C)				0.0063	0.0129
Rd KW20 *mef*(A)					0.0099
Rd KW20 *mef*(E)					

*A p-value of 0.05 was used as the significance cut-off

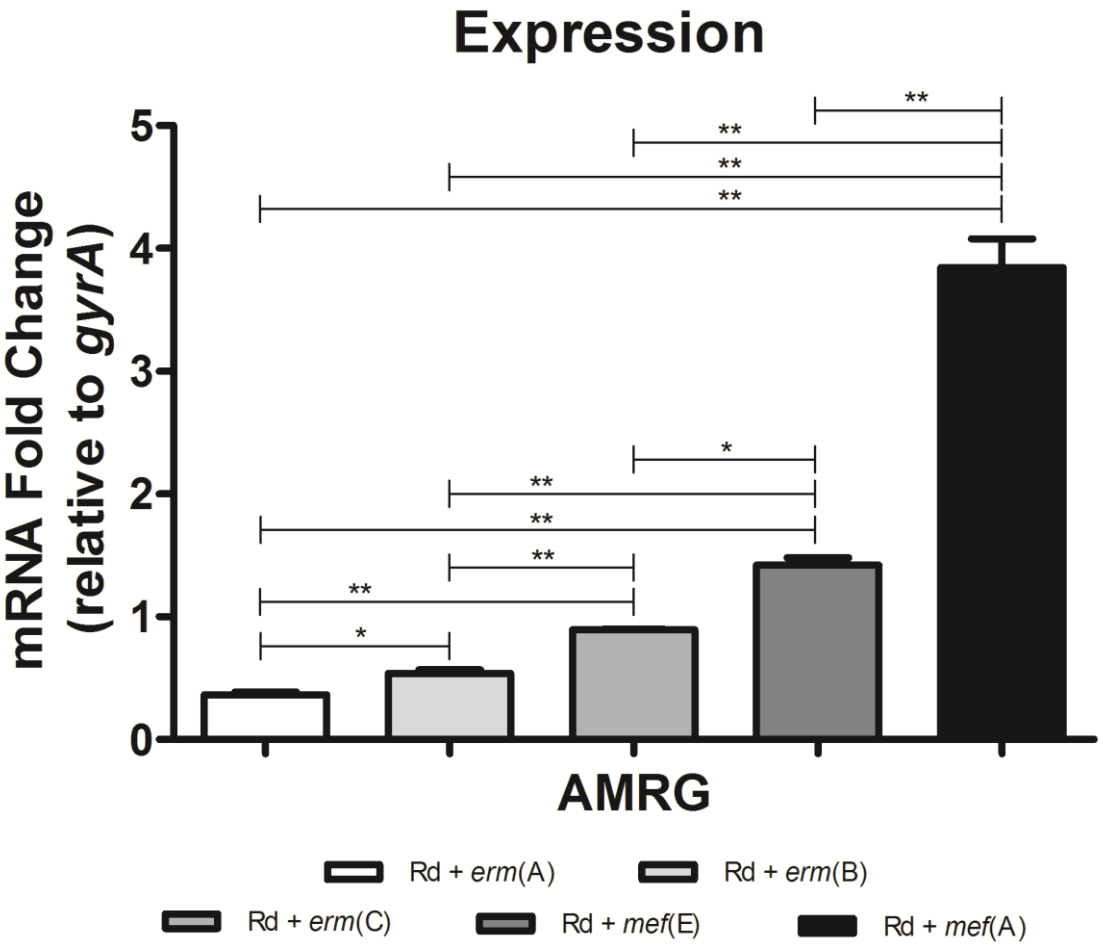

Figure 6.2: ΔCT values (mRNA Fold Change (relative to *gyr*A)) for each of the 5 transformants ("no native promoter"). Each level of expression of each AMRG was statistically significantly different when compared to one another.

*, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

Table 6.5: Erythromycin and azithromycin MIC results for untransformed *H. influenzae* Rd KW20 and each transformant ("no native promoter").

Strain	**MIC (µg/mL)**	
	AZ	**ERY**
H. influenzae Rd KW20 (no insert)	4	8
H. influenzae Rd KW20 (*erm*(A))	4	16
H. influenzae Rd KW20 (*erm*(B))	32	64
H. influenzae Rd KW20 (*erm*(C))	>64	>64
H. influenzae Rd KW20 (*mef*(A))	4	8
H. influenzae Rd KW20 (*mef*(E))	4	8

discussed, Roberts et al. did not analyse these transconjugants for alternate resistance mechanisms such as L4, L22 and 23S rRNA alterations.[93] If these transconjugants did have an alternate mechanism or mutation that had developed in the background during conjugation, it may be that this is contributing to the phenotype (either in combination with the effect of *mef* or as the sole contributor). During our study, we used media supplemented with kanamycin to select for transformants rather than a macrolide; given that our transformants were not exposed to the same selective pressure present in the Roberts study to select background mutants, it is probable that our transformants lack this additional mechanism required for *mef* to have an effect on the macrolide resistance phenotype. This may also explain difference between our study and the observations of Tinguely et al. (2011), in which isolates of *H. parainfluenzae* exhibiting high-level macrolide resistance were found to carry *mef*(A) but also bore the macrolide resistance-associated L4 alteration A69S that may have also contributed to the phenotype in those particular isolates.

Another explanation for our observations is that our study did not account for the role of *msr*(D), an *msr*(A) homolog found downstream of the *mef* gene. Previous studies have indicated that *mef* and *msr*(D) are co-transcribed as a inducible dual efflux system,[184,185] and that *msr*(D) may be critical for the development of the M phenotype.[186,187] The inserts prepared for the clones generated in this study did not cover the range including *msr*(D). Given that the isolates of *H. influenzae* and *H. parainfluenzae mef* genes detected by Roberts et al. and Tinguely et al., respectively, were naturally occurring and likely carried *msr*(D) alongside *mef*, the presence of *msr*(D) in those isolates (and subsequently generated transconjugants) may have also contributed to the difference in the phenotype in our transformants. While the role of *msr*(D) is beyond the scope of this particular study, there is merit in investigating the role of *msr*(D), both standalone and in unison with *mef*, in the development of macrolide resistance in NTHi.

After this initial experiment, we then attempted to clone each of these AMRGs with their respective native promoters to determine if any change in phenotype and expression level was apparent. ΔCT values were obtained for each of these new "native promoter" transformants and compared to the corresponding "no native promoter" transformants obtained in the first experiment. Of the 5 genes included in this study, the level of expression of the *erm*(B) and *mef*(E) transformants did not appear to be statistically significantly different between the two pairs (*erm*(B), p=0.6186; *mef*(E), p=0.3746; see Figure 6.3). When the erythromycin and azithromycin MICs were determined for the *erm*(B) and *mef*(E) "native promoter" transformants, no significant changes in the MICs were apparent compared to the corresponding "no native promoter" transformants (see Table 6.6), which is consistent with the findings of the expression analyses. On the other hand, the *erm*(C) and *mef*(A) "no native promoter" transformants produced a significantly lower ΔCT than the corresponding "no native promoter" transformant (*erm*(C), p=0.0013; *mef*(A), p=0.0049; see Figure 6.3). In the case of the *erm*(C) transformant, this drop in expression resulted in a corresponding decrease in erythromycin and azithromycin MICs compared to its paired "no native promoter" transformant (see Table 6.6), so we can conclude that this drop in expression was sufficient to result in a less resistant phenotype in *H. influenzae*. No changes in erythromycin and azithromycin MICs were observed in the *mef*(A) transformant. Given that the expression observed in the *mef*(A) "no native promoter" transformant was found not to be sufficient to increase the MICs in *H. influenzae*, this particular observation is not unexpected.

The *erm*(A) "native promoter" transformant produced a significantly higher ΔCT than its corresponding "no native promoter" transformant (*erm*(A), p=0.0097; see Figure 6.3). In addition, a corresponding and significant increase in erythromycin and azithromycin MICs of the *erm*(A) "native promoter" transformant was observed (see Table 6.6). From this, we can

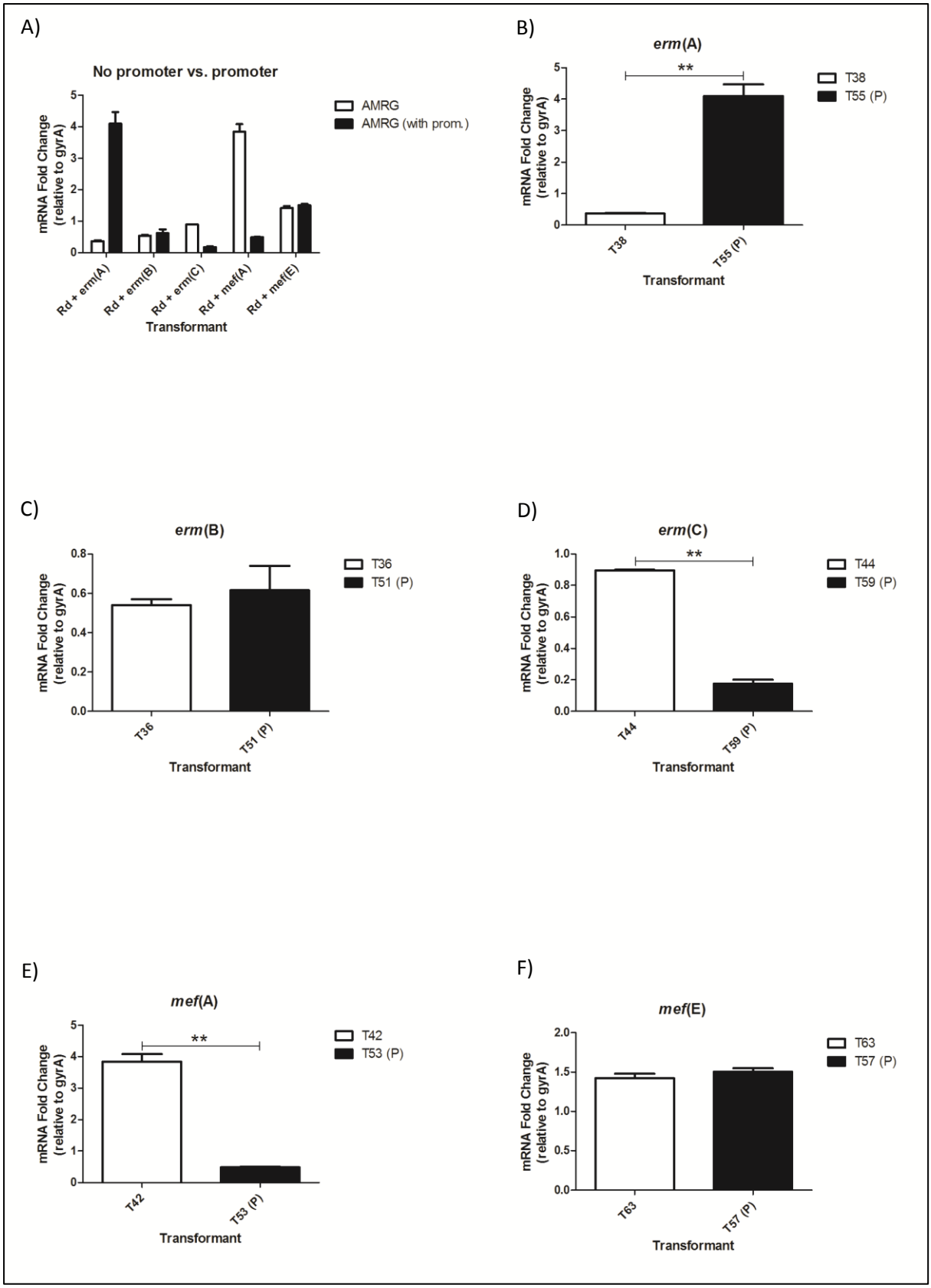

Figure 6.3: ΔCT values (mRNA Fold Change (relative to *gyr*A)) for each of the 5 transformants ("no native promoter" vs. "native promoter"). A, all genes; B, *erm*(A); C, *erm*(B); D, *erm*(C); E, *mef*(A); F, *mef*(E).

*, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

Table 6.6: Erythromycin and azithromycin MIC results for each transformant ("native promoter").

Strain	MIC (µg/mL)	
	AZ	ERY
H. influenzae Rd KW20 (*erm*(A))	>64	>64
H. influenzae Rd KW20 (*erm*(B))	>64	>64
H. influenzae Rd KW20 (*erm*(C))	4	16
H. influenzae Rd KW20 (*mef*(A))	4	8
H. influenzae Rd KW20 (*mef*(E))	4	8

say that this increase in expression was enough to generate a resistant phenotype. It is interesting to note that the level of expression of *erm*(A) required for the development of macrolide resistance appears to be greater than the level of expression of *erm*(B) and *erm*(C) required to give a similar outcome. As referred to previously, this may indicate that *erm*(B) and *erm*(C) produce greater efficiency in modifying the macrolide binding target site, although further investigations would be required to establish this. It should be noted here that while this study involved the analysis of gene expression through cDNA measurements, no measurements of protein levels were made. Given that there may be potential for post-transcriptional regulation of these resistance genes, it would be valuable to additionally analysis protein levels to see if the differences in the various *erm* genes attributable to factors other than gene expression. In any case, expression of all *erm* genes of interest and subsequent development of macrolide resistance in *H. influenzae* has been demonstrated across these two studies.

As previously discussed in this thesis, AMRGs can be inducibly or constitutively expressed depending on the structure of the promoter, and among our collection of donors, the *erm*(A) and *erm*(C) donors (U37 and U387, see Table 6.1) were found to be inducibly resistant to clindamycin by D test. The *erm*(B) (U200) donor was constitutively resistant to clindamycin by comparison. To see if this profile was retained by the "native promoter" transformants, we performed D tests on each of these *erm* gene transformants, using untransformed *H. influenzae* Rd KW20 as a control. No evidence of induction was found in any of these transformants. The *erm*(C) transformant D test profile was identical to the untransformed *H. influenzae* Rd KW20 control, suggestive of no reduced MLS_B antibiotic susceptibility. Given that the previous expression analyses had shown that the "native promoter" *erm*(C) transformant had reduced expression of *erm*(C) (see Figure 6.3) and that the erythromycin and azithromycin MICs were not increased significantly compared to the untransformed

control (see Table 6.6), the D test results is not surprising. The D test results for the *erm*(A) and *erm*(B) transformants indicated that the genes were being constitutively expressed (no zones of inhibition, see Table 6.7). In the case of *erm*(A), this change from inducible phenotype (in the original donor) to constitutive phenotype (in the transformant) may be reflective of the high degree of expression of *erm*(A) observed in the transformant in the absence of the inducer (see Figure 6.3).

It is important to note that the D test performed here is primarily designed for use for the detection of inducible clindamycin resistance in *S. aureus* and other Gram positive cocci, and it is not optimised for use in *H. influenzae*. When performing the tests here, the transformants were tested on chocolate agar and grown overnight in a CO_2 environment, and it is not clear what impact these factors have on the performance of the test. In addition, the erythromycin and clindamycin discs were placed 15 mm apart in accordance with previously described methodologies but it is not known if this is the optimal spacing for this species. Given there is precedent for the importance of proper disc spacing in antibiotic resistance testing of *H. influenzae*, namely in regards to disc spacing issues presented in the detection of extended-spectrum β-lactamases (ESBLs) in *H. influenzae* through adjacent positioning of cephalosporin and augmentin discs,[292] we felt an alternative method for testing the effect of inducers on the expression of the AMRGs in our "native promoter" transformants was warranted. Therefore, we decided to repeat the expression analysis on the "native promoter" transformants (including the *mef* transformants), but with growth of the transformants in broth supplemented with a sub-inhibitory dose of erythromycin, a known inducing agent of

these genes, to see if expression would be increased in any of our transformants under these conditions. When performing these assays, we chose to supplement our growth media with erythromycin set at a concentration of 0.125 of the erythromycin MIC of the transformant of interest, and we should note here that little information is available on what would be an

Table 6.7: D test results for *erm* transformants ("native promoter").

Strain	E15 zone size* (mm)	DA2 zone size* (mm)	D Test Interpretation
H. influenzae Rd KW20	12	10	-
H. influenzae Rd KW20 (*erm*(A))	0	0	- (constitutive)
H. influenzae Rd KW20 (*erm*(B))	0	0	- (constitutive)
H. influenzae Rd KW20 (*erm*(C))	12	11	-

* Diameter of inhibition around disc

appropriate concentration of erythromycin for this kind of investigation in *H. influenzae* and that the selection of this particular concentration was somewhat arbitrary. One previous study investigating induction of AMRGs in *S. pneumoniae* used concentrations of inducer set at 0.1-0.2 of the MIC of the strain,[293] so the selection of 0.125 in the present study has some precedent, but to further investigate the effect of inducers on the expression of AMRGs in *H. influenzae*, there may be some merit in performing the expression analyses using a range of different concentrations of the inducer to determine if the effect changes depending on this variable.

ΔCT values were obtained for each transformant and compared to the corresponding ΔCT from the "native promoter" transformants grown without the inducer. The ΔCT values for *erm*(A), *erm*(C) and *mef*(E) appeared to be increased after growth with the presence of erythromycin, with a slight drop for *mef*(A) and little difference for *erm*(B). However, for each of the 5 genes, these differences did not appear to be statistically significantly different (*erm*(A), p=0.0736; *erm*(B), p=0.8586; *erm*(C), p=0.4693; *mef*(A), p=0.4597; *mef*(E), p=0.3483; see Figure 6.4). Based on the findings of this study we are unable to conclude that the inducer has truly had an effect on the expression of these AMRGs in *H. influenzae*, and as such we have not been able to demonstrate potential inducible clindamycin resistance in our transformants.

To summarise this chapter, we reiterate that the main aim of this chapter was to investigate the phenotypic effect of expression of select AMRGs in *H. influenzae*, using a number of different approaches. Through cloning of these genes into a shuttle vector and subsequent transformation of these vectors into *H. influenzae* Rd KW20, our findings indicate that

expression of each of *erm*(A), *erm*(B) and *erm*(C) can result in a macrolide resistant phenotype in *H. influenzae*, with the latter two being potentially more efficient than *erm*(A) in this regard. On the other hand, expression of *mef*(A) and *mef*(E) did not

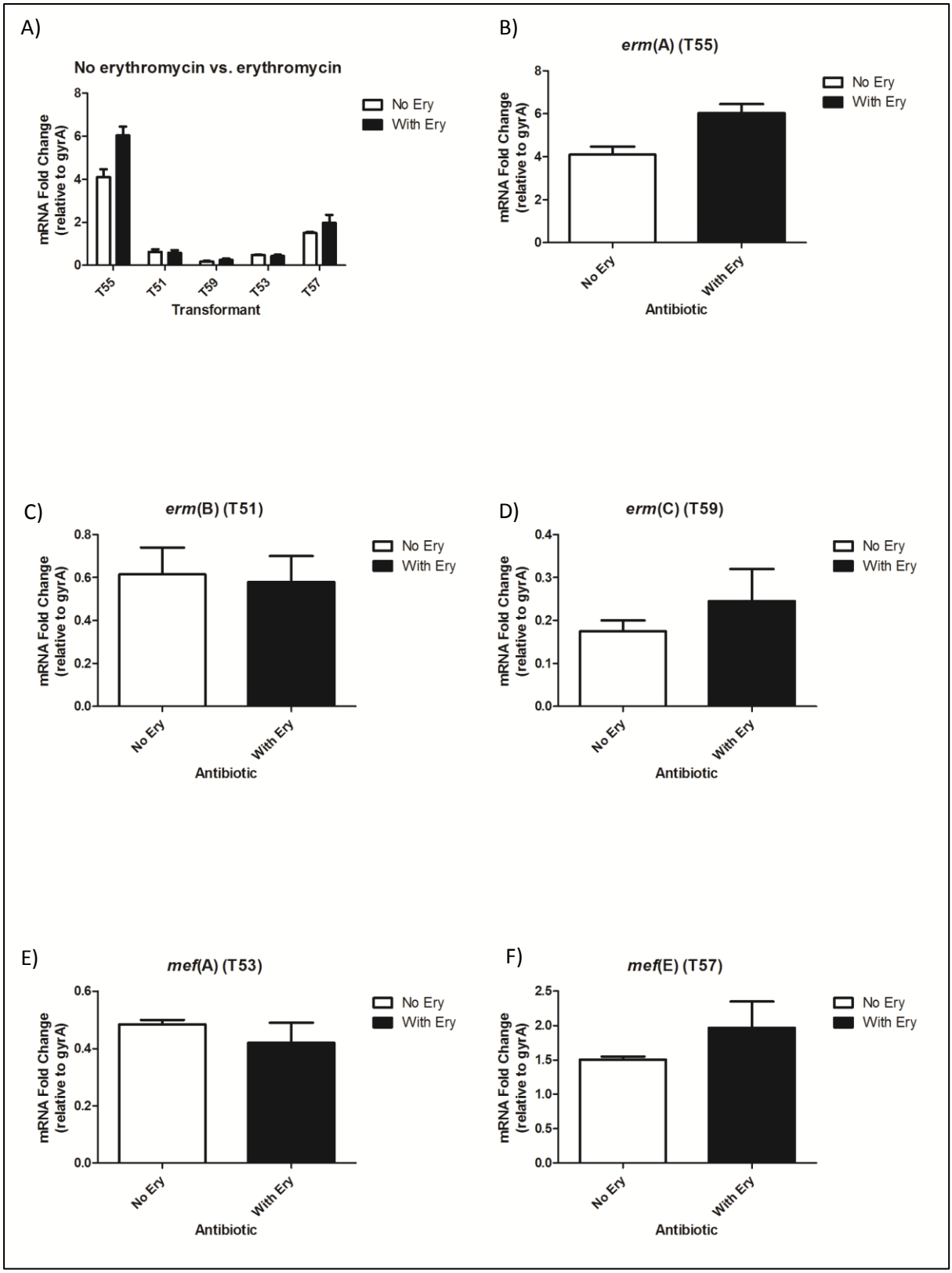

Figure 6.4: ΔCT values (mRNA Fold Change (relative to *gyr*A)) for each of the 5 transformants ("native promoter"; "no erythromycin" vs. "erythromycin"). A, all genes; B, *erm*(A); C, *erm*(B); D, *erm*(C); E, *mef*(A); F, *mef*(E).

*, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

appear to have an effect on the macrolide resistance phenotype in this species on their own. Given that Roberts et al. (2011) have previously demonstrated that isolates acquiring *mef* can develop macrolide resistance, our findings indicate that these genes may only produce an effect in combination with another mechanism, such as underlying chromosomal mutations, or *msr*(D). Further work is required to establish just how these different resistance mechanisms interact with one another.

It is still unclear how much of a threat these particular AMRGs pose to future treatment regimes of diseases involving NTHi. While we have been able to demonstrate a resulting high-level resistance phenotype through expression of *erm*(A), *erm*(B) and *erm*(C) in the present study, we were unable to demonstrate conjugative transfer of these *erm* or *mef* genes to *H. influenzae* and have yet to find these genes in wild-type strains of NTHi. The study of Roberts et al. (2011) remains the only study in which these genes have been reported in wild-type strains of NTHi, and while the authors of that study were able to demonstrate conjugative transfer of *mef*(A), *erm*(F) and *erm*(A), the MICs in resulting transconjugants was only moderately increased and cannot definitively be attributed to the presence of these genes alone.[93] The potential role of these and other AMRGs in NTHi may warrant further monitoring in the future due to the potential for the acquisition and expression of these genes to cause reduced macrolide susceptibility, but based on the information we have currently acquired, we conclude that AMRGs are not a widespread threat among NTHi isolates and that chromosomal alterations remain the main contributor to high-level macrolide resistance acquisition in this species.

Chapter 7: Conjugative transfer of select acquired macrolide resistance genes from *Pasteurella multocida* to *Haemophilus influenzae*

7.1 Introduction

The bacterial family *Pasteurellaceae* covers a range of gram negative bacteria that typically act as upper respiratory commensals in a variety of mammal and bird species. Genera included within this family include *Haemophilus*, *Pasteurella* and *Mannheimia*. Members of this family have been shown to be capable of exchanging genetic material and this has facilitated the exchange of antibiotic resistance determinants.

A number of acquired macrolide resistance genes (AMRGs) have been detected among animal commensals that have been implicated as opportunistic pathogens, such as *Mannheimia* and *Pasteurella*. In particular, the ARMGs *erm*(42), *msr*(E) and *mph*(E) have previously been reported among field isolates of *Pasteurella multocida* and *Mannheimia haemolytica* and have been associated with resistance to 14-, 15- and 16-membered macrolides (and closely related antibiotics such as tulathromycin). The resulting phenotype appears to depend on whether the genes exist alone or are present in particular combinations with one another. Isolates carrying *erm*(42) alone typically produce moderate resistance to macrolides and high-level resistance to lincosamides, while isolates carrying *msr*(E) and *mph*(E) produce resistance against 14- and 15-membered macrolides and tulathromycin, but comparatively less resistance against lincosamides and 16-membered macrolides. In addition, isolates simultaneously carrying all three and exhibiting high-level resistance to MLS_B antibiotics have also been reported.[265]

Recently, a mobile, multiresistance integrative conjugative element (ICE), designated ICE*Pmu1*,[263,264] has been described in a bovine *Pasteurella multocida* isolate and was found to carry a large number of resistance determinants, including *erm*(42), *msr*(E) and *mph*(E). The structure of ICE*Pmu1*, reportedly the first ICE in this species, has been deduced. ICE*Pmu1*, 82214 bp in size and harbouring 88 reading frames, contains a core set of genes that are involved in excision, integration and conjugative transfer, and that demonstrate a great degree of similarity with alternative ICEs found in the bovine respiratory pathogens *M. haemolytica* and *Haemophilus somni*. The ICE carries 12 resistance genes organised into 2 separate resistance regions (see Figure 7.1) located 42526 bp apart. These regions exhibit extensive similarities with transposons, plasmids and other elements previously identified in different species, indicating that they came about as a result of a series of integration and recombination events in which insertion sequences have played a significant role. The first region, 15711 bp in size, contains *erm*(42), along with resistance markers against kanamycin and neomycin (*aphA1*), streptomycin (*strA* and *strB*), sulfonamides (*sul2*), and chloramphenicol and florfenicol (*floR*). The second region, 9789 bp in size, contains *msr*(E) and *mph*(E) in addition to resistance markers against gentamicin (*aadB*), spectinomycin and streptomycin (*aadA25*), ampicillin (bla_{OXA-2}) and tetracycline (*tet*(H)). Conjugative experiments have shown that ICE*Pmu1* is an active element that can be transferred to other *P. multocida* isolates as well as to *M. haemolytica* and *Escherichia coli*. Increases in MICs of the above antibiotics, including against clindamycin, tilmicosin and tulathromycin, were observed in resulting transconjugants. In all of these experiments, ICE*Pmu1* was integrated into a $tRNA^{Leu}$ site;[264] this site has also been recognised as a common integration site for resistance elements in *H. influenzae*, particularly β-lactamase-harbouring genomic islands such as ICE*Hin1056*.[294,295]

It has previously been demonstrated that *Pasteurella* and *Haemophilus* are capable of sharing

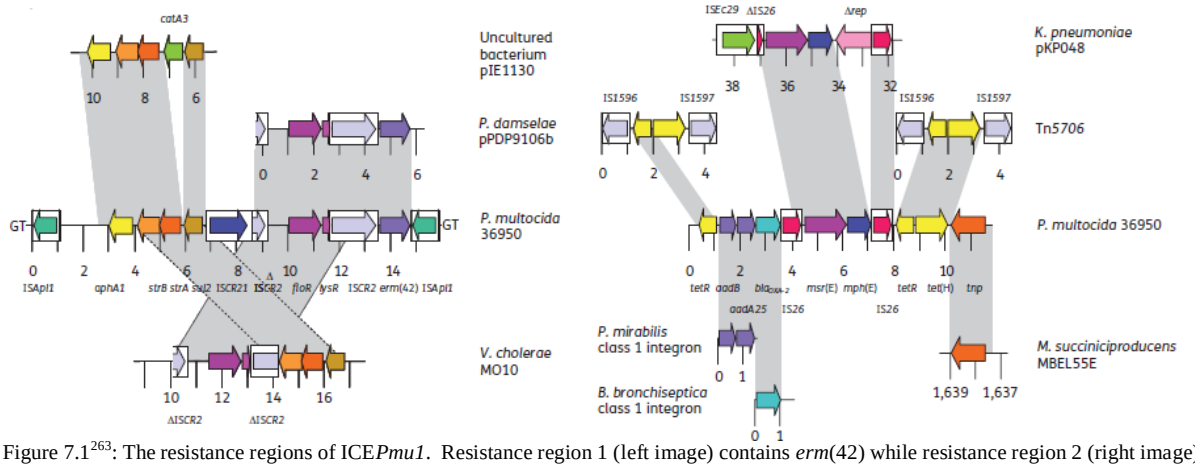

Figure 7.1[263]: The resistance regions of ICE*Pmu1*. Resistance region 1 (left image) contains *erm*(42) while resistance region 2 (right image) contains *msr*(E) and *mph*(E).

resistance determinants. For example, the bla_{ROB-1}-carrying plasmid pB1000, first described in *H. parasuis* in swine[266] and subsequently reported in animal-derived *P. multocida* isolates, has also been reported in human clinical *H. influenzae* isolates in Spain and Australia.[262,268] Indeed, previous reports have demonstrated that approximately 5% of β-lactamase positive *H. influenzae* strains carry a bla_{ROB-1}-type β-lactamase worldwide[268] (with the majority found in the United States and Mexico),[296] and evidence suggests that the majority of these bla_{ROB-1}-type β-lactamases in *H. influenzae* are carried on pB1000-related elements.[262,266,268] Given the previous detection of AMRG-carrying conjugative elements such as ICE*Pmu1* in *P. multocida*, there is precedent for the possible transfer of AMRG-bearing mobile elements and the associated phenotypic resistance from *P. multocida* to NTHi. The aim of this study was to look for evidence of the AMRGs *erm*(42), *msr*(E) and *mph*(E) in wild-type strains of NTHi and demonstrate *in vitro* conjugative transfer of these AMRGs on ICE*Pmu1* from *P. multocida* to *H. influenzae*, and to investigate the phenotypic effect of these genes in *H. influenzae*.

7.2 Methods and Materials

7.2.1 Detection of *erm*(42), *msr*(E) and *mph*(E) in clinical isolates of *Haemophilus influenzae*

Before commencing the main component of this study, we screened a number of NTHi isolates for the presence of *erm*(42), *msr*(E) and *mph*(E) to detect any evidence of transfer of these genes to NTHi from *Pasteurella* and other genera that act as reservoirs for these genes. The isolate collection included in this screen consisted of 145 clinical respiratory isolates of NTHi identified by colony morphology, X and V factor dependence and a positive PCR for either *fucK* or *hpd* as previously described.[297] Of these, 59 isolates were from CF patients, and the remaining 86 isolates were from a range of other patients. The previously determined azithromycin MICs of this collection ranged from 0.09->256 µg/mL, with MIC_{50} and MIC_{90} values of 1 µg/mL and 3 µg/mL, respectively. All isolates were cultured on chocolate agar.

A previously described multiplex PCR system was used for the detection of *erm*(42), *msr*(E) and *mph*(E).[298] Primers used in this reaction can be found in Table 7.1.

7.2.2 Conjugation of ICE*Pmu1* to *Haemophilus influenzae* Rd KW20

To transfer ICE*Pmu1* to *H. influenzae*, mating experiments were performed using *P. multocida* strain 36950 (GenBank accession CP003022.1),[263] a bovine-derived *P. multocida* isolate carrying ICE*Pmu1*, as the donor and a previously generated nalidixic-resistant isolate of *H. influenzae* Rd KW20 as the recipient. The donor and recipient were mixed in a ratio of 1:5 and transconjugants were grown on chocolate agar with tetracycline (15 µg/mL) and nalidixic acid (8 µg/mL). Transfer efficiency was calculated as the number of transconjugant CFUs per recipient CFU. Refer to Chapter 3 for further information.

Table 7.1: Primers used for the detection of *erm*(42), *msr*(E) and *mph*(E) in *H. influenzae*.

Gene/Region	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence
erm(42)	Detection of *erm*(42)	59	p64[298]	TGCACCATCTTACAAGGAGT
			p66[298]	CATGCCTGTCTTCAAGGTTT
mph(E)	Detection of *mph*(E)		p67[298]	ATGCCCAGCATATAAATCGC
			p68[298]	ATATGGACAAAGATAGCCCG
msr(E)	Detection of *msr*(E)		p70[298]	TATAGCGACTTTAGCGCCAA
			p71[298]	GCCGTAGAATATGAGCTGAT

7.2.3 Characterisation of ICE*Pmu1* in transconjugants

To confirm the identity of any transconjugants as ICE*Pmu1*-carrying *H. influenzae*, PCRs specific for *Haemophilus* spp. (P6) and *P. multocida* (*kmt-1*), as well as PCRs for the detection of the integrase and relaxase genes of ICE*Pmu1* were performed. Further information and primer sequences are provided in Table 7.2. Isolates identified as ICE*Pmu1*-carrying *H. influenzae* underwent further PCR testing to determine which elements of ICE*Pmu1* were transferred. PCRs were run for the detection of the individual resistance genes (including *erm*(42), *msr*(E) and *mph*(E)) as well as for the detection of linkages between particular elements of ICE*Pmu1*. Further details and primer sequences are provided in Tables 7.3 and 7.4.

7.2.4 Antibiotic resistance testing

MICs of ICE*Pmu1*-relevant antibiotics were determined by broth microdilution, as previously described in Chapter 3, on any ICE*Pmu1*-carrying *H. influenzae* isolates (as confirmed by PCR). Antibiotics included in the panel were gentamicin, neomycin, tetracycline, tilmicosin, tulathromycin, chloramphenicol, florfenicol, streptomycin, ampicillin, erythromycin, clindamycin, spectinomycin, kanamycin and sulfamethoxazole.

7.2.5 Retention of transfer functions of ICE*Pmu1* in *Haemophilus influenzae*

To demonstrate that the transfer functions of ICE*Pmu1* were retained in *H. influenzae*, mating experiments were performed using an *H. influenzae* Rd KW20 carrying ICE*Pmu1* previously generated by conjugation assay with *P. multocida* 36950 as a donor and a separate rifampicin-resistant and nalidixic-susceptible *H. influenzae* Rd KW20 as a recipient. The donor and recipient were mixed in a ratio of 1:10 and transconjugants were grown on chocolate agar with kanamycin (30 µg/mL) and rifampicin (50 µg/mL). Transfer efficiency

Table 7.2: Primers used for the detection of ICE*Pmu1*-carrying *H. influenzae*.

Gene/Region	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence
kmt-1	*P. multocida*-specific	55	KMT1SP6[299]	GCTGTAAACGAACTCGCCAC
			KMT1T7[299]	ATCCGCTATTTACCCAGTGG
P6	*Haemophilus* spp.-specific	54	P6-fwd[300]	ACGATGCTGCAGGCAATGGT
			P6-rv[300]	CATCAGTATTACCTTCTACTAAT
ICE_2051	Detection of the relaxase gene of ICE*Pmu1*	60	ICE-relaxase-fw[264]	CTGGTTCAACGTCCTGTCAA
			ICE-relaxase-rv[264]	ATCGTTGCAATTTCCTGTCC
ICE_2052	Detection of the integrase gene of ICE*Pmu1*	60	ICE-integrase2052-fw*	TGGCGTAATCAAATTGTTGG
			ICE-integrase2052-rv*	AGCTGATTTTGGCTCATTGG

*Primers are unpublished

Table 7.3: Primers used for the characterisation of resistance region 1.

Gene/Region	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence
aphA1	Detection of the kanamycin and neomycin resistance gene *aphA1*	62	aph(3')I-B[301,302]	GGCAAGATCCTGGTATCGGTCTGC
			aph(3')I-F[301,302]	AACGTCTTGCTCGAGGCCGCG
aphA1/*strA*	Detection of the genetic linkage of *strA* and *aphA1*	55		
strA/*strB*	Detection of the genetic linkage of the streptomycin resistance genes *strA* and *strB*	55	str1[303]	TGACTGGTTGCCTGTCAGAGG
			strB-R[304]	GGATCGTAGAACATATTGGC
ICE_3	Detection of the genetic linkage of *strA* and IS*CR21*	59	ICE_past3-fw*	GCAAACAAACGGGTCAACTT
			str2[303]	CCAGTTGTCTTCGGCGTTAGCA
sul2/*strA*	Detection of the genetic linkage of sul2 and strA	57		
sul2	Detection of the sulfonamide resistance gene *sul2*	67	sul1[303]	ACAGTTTCTCCGATGGAGGCC
			sul2[303]	CTCGTGTGTGCGGATGAAGTC
ICE_4	Detection of the genetic linkage of IS*CR21*and *floR*	56	ICE_past4-fw*	CTGTGACCAAAAACGGACCT
			ICE_past4-rv*	CGGTAGGATGAAGGTGAGGA
floR	Detection of the florfenicol resistance gene *floR*	55	floR-$_{STOP}$[305]	CGGTTAGACGACTGGCGACT
			floR-$_{START}$[305]	AGGGTTGATTCGTCATGACCA
floR/*erm*(42)	Detection of the genetic linkage of *floR* and *erm*(42)	55		
			erm(42)-fw[230]	ACGTTGCACTTGGTTTGACA
erm(42)	Detection of the macrolide-lincosamide resistance gene *erm*(42)	55	Pm36950-erm-fw*	ATCTGCAAAGCCGTTAATGC
			Pm36950-erm-rv*	ATCCTTGCTTACCATGTTCG
ICE_5	Detection of the genetic linkage of IS*CR2* and IS*Apl1*	60	ICE_past5-fw*	TACCGAAGCCCAGAGTCAAC
			ICE_past5-rv*	ACGTAGCTCCAGCACCATTT

*Primers are unpublished

Table 7.4: Primers used for the characterisation of resistance region 2.

Gene/Region	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence
ICE_1	Detection of the genetic linkage of *tetR* and IS*26*	55	ICE_past1-fw*	TTCACGCTCTTTTTGGCTTT
			ICE_past1-rv*	ACCTTTGATGGTGGCGTAAG
aadB	Detection of the gentamicin resistance gene *aadB*	57	ant(2")-I-B[301,306]	TATCGCGACCTGAAAGCGGC
			ant(2")-I-F[301,306]	GGGCGCGTCATGGAGGAGTT
aadB- bla_{oxa-2}	Detection of the genetic linkage of *aadB* and bla_{OXA-2}	57		
bla_{oxa-2}	Detection of genes coding for beta-lactamases of OXA-2 group	60	bla_{oxa-2}-rev[307]	CCACTCAACCCATCCTACCC
			bla_{oxa-2}-fw[307]	AAGAAACGCTACTCGCCTGC
ICE_6	Detection of the genetic linkage of bla_{OXA-2} and *msr*(E)	58		
msr(E)	Detection of the macrolide resistance gene *msr*(E)	57	Pm36950-mel-rv*	CTGCCGTAATCGGGAACTTA
			Pm36950-mel-fw*	TATCTCGCCTTGGTGTTTCC
aadA25	Detection of variants of the streptomycin and spectinomycin resistance gene *aadA*	60	ant(3')I-F[301,308]	GTGGATGGCGGCCTGAAGCC
			ant(3')I-B[301,308]	ATTGCCCAGTCGGCAGCG
mph(E)	Detection of the macrolide resistance gene *mph*(E)	57	Pm36950-mph-rv*	GGGTGAAATCTGCCCATAGA
			Pm36950-mph-fw*	ATCACTTGCTGAAGCACACG
ICE_7	Detection of the genetic linkage of *mph*(E) and *tet*(H)	57		
tet(H)	Detection of the tetracycline resistance gene *tet*(H)	55	tet(H)-2[309]	TCCCAATAAGCGACGCT
			tet(H)-1[309]	ATACTGCTGATCACCGT
msr(E)-*mph*(E)	Detection of the genetic linkage of *msr*(E) and *mph*(E)	55	msr(E)-fw[230]	TACCGGAACAACGTGATTGA
			mph(E)-rv[230]	GAAGGGTTACGCCAGTACCA
ICE_2	Detection of the genetic linkage of IS*26* and *tnp*	59	ICE_past2-fw*	CAACGTGAAGAAGTGGCAGA
			ICE_past2-rv*	ACCGCCTGATCCAGTACATC

*Primers are unpublished

was calculated as previously stated. PCR was used to confirm transfer as described above. To confirm CFUs as true transconjugants and not simply rifampicin-resistant mutant donors, isolates were tested for rifampicin and nalidixic acid resistance by disk diffusion in accordance with CDS methodology, using the donor and recipient as controls. Transconjugants were confirmed by matching their rifampicin and nalidixic acid resistance profiles with that of the recipient.

Additional mating experiments were also performed using 2 rifampicin-resistant NTHi strains (*H. influenzae* NCTC 4560, and a separate clinical isolate (Ci6)) as recipients in an attempt to demonstrate conjugative transfer of ICE*Pmu1* to different strains of *H. influenzae*. A range of donor:recipient ratios (1:1, 1:5 and 1:10) across different experiments were used.

7.2.6 Determination of insertion point of ICE*Pmu1* into *Haemophilus influenzae*

To establish the insertion point of ICE*Pmu1* into the *H. influenzae* genome, we designed primers for the amplification of $tRNA^{Leu}$ sites, the expected insertion site of ICE*Pmu1*. Four primer sets (see Table 7.5 for sequences) were used to amplify separate $tRNA^{Leu}$ sites in *H. influenzae* Rd KW20 (as indicated on reference sequence NC_000907.1 on GenBank). Primers were designed using Primer-BLAST (National Center for Biotechnology Information, USA); *H. influenzae* Rd KW20 (GenBank accession NC_000907.1) was used as the reference template. Our donor *P. multocida* (with ICE*Pmu1*) and recipient *H. influenzae* Rd KW20 strains were used as negative and positive controls for these PCR reactions, respectively.

Table 7.5: Primers for the determination of the insertion point of ICE*Pmu1* in *H. influenzae*.

Gene/Region	Description of PCR	Annealing Temp. (°C)	Primer Name	Primer Sequence
HI0123	Amplification of the $tRNA^{Leu}$ site HI0123 and its flanking region.	55	HI0123_F	TCCGTAAAATACGCCCCGTT
			HI0123_R	GGGCGATGTAGAAGAAGCGA
HI0086	Amplification of the $tRNA^{Leu}$ site HI0086 and its flanking region.	55	HI0086_F	CTCATCACTGCGATTGCCTG
			HI0086_R	TGCACAACTACGGGCTTACT
HI1631	Amplification of the $tRNA^{Leu}$ site HI1631 and its flanking region.	57	HI1631_F	TTTCGACAAGCCTCCCGTTT
			HI1631_R	CACAAGCTGTCATCGCATCG
HI1424	Amplification of the $tRNA^{Leu}$ site HI1424 and its flanking region.	55	HI1424_F	TCTTTTCTTCTTCCGCACCC
			HI1424_R	CCCTGATTGAGCTCCTGCAA

7.2.7 Whole genome sequencing of transconjugants

Select transconjugants were submitted for whole genome sequencing. In addition to providing further confirmation of the presence of ICE*Pmu1* and its various components, including *erm*(42), *msr*(E) and *mph*(E), whole genome sequencing also allowed for the confirmation of the insertion point of ICE*Pmu1* indicated by the above PCRs, and also served as an alternative way to determine the insertion point should these PCRs indicate that it may be in an alternative site to those investigated above.

A total of 6 transconjugants were submitted for whole genome sequencing. Three of these (T65, T69 and T79) were primary transconjugants derived from two separate conjugation assays between the *P. multocida* donor and the *H. influenzae* recipient as described in section 7.2.2. The other three (A27, A28 and A37) were secondary transconjugants derived from two separate conjugation assays between the ICE*Pmu1*-carrying *H. influenzae* Rd KW20 generated in this study and a separate *H. influenzae* Rd KW20 recipient as described in section 7.2.5 (all three A transconjugants are *H. influenzae* Rd KW20). Transconjugants were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Australia).

Sequences underwent quality checks as previously described in Chapter 3 of this thesis. After all Contigs were assembled, Contigs of a length less than 1000 bp were filtered out. The filtered Contig files were then ordered against the reference *H. influenzae* Rd KW20 genome on GenBank (GenBank no.: NC_000907.1) using the Mauve Contig Mover.[310] The ordered Contigs were joined into a single fasta sequence using the Artemis genome browser.[311] The joined sequences were aligned to the *H. influenzae* Rd KW20 genome and visualised as a ring image using the BLAST Ring Image Generator (BRIG).[312]

The joined fasta files were uploaded to CLC Main Workbench 7 for visual inspection and analysis to detect the presence of ICE*Pmu1* in the transconjugants and to determine the integration site of the ICE into the *H. influenzae* Rd KW20 genome.

7.2.8 Effect on fitness of ICE*Pmu1* and ICE*Pmu1* stability

The fitness cost of ICE*Pmu1* was determined by competition experiments between *H. influenzae* Rd KW20 and *H. influenzae* Rd KW20/ICE*Pmu1*, adapted from a protocol previously described by San Millan et al. (2015),[313] in sBHI (refer to Chapter 3 for further information). Briefly, strains were grown overnight on chocolate agar, and then 10^6 CFU of *H. influenzae* Rd KW20 were mixed with 10^6 CFU of *H. influenzae* Rd KW20/ICE*Pmu1* in 2 mL of sBHI. This mix was grown at 37°C and 150 rpm for 24 hours, and 10^6 CFU was subsequently transferred to 2 mL of fresh sBHI every 24 hours (1/1000 dilution, or 2 µL of growth in fresh sBHI to a total volume of 2 mL). This was done for 6 consecutive days; samples were collected at time zero and every 24 hours thereafter. For each sample, aliquots were plated on non-selective chocolate agar, and the proportion of resistant colonies was then estimated by plating of 100 colonies on chocolate agar plates containing 30 µg/mL of kanamycin. At each 24 hour time point, the ratio of resistant vs. susceptible colonies was determined by dividing the number of CFUs with ICE*Pmu1* by 50 (50 is the expected CFU count if the number of cells with ICE*Pmu1* was equal to the number of cells without ICE*Pmu1*). As an additional measure of fitness, the selection coefficient was calculated as previously described.[313] To control for selection bias, five replicate experiments were performed, and the average ratio across these replicates was determined at each 24 hour time point.

The stability of ICE*Pmu1* in *H. influenzae* was determined in a similar experiment to that described above, but with *H. influenzae* Rd KW20/ICE*Pmu1* growing alone rather than in competition with *H. influenzae* Rd KW20. Three replicate experiments were performed.

7.3 Results and Discussion

The initial screen for the detection of *erm*(42), *msr*(E) and *mph*(E) failed to detect evidence of any of these genes in our collection of NTHi respiratory isolates. Given that we had also failed to detect these genes in any of the whole genome sequences included in the AMRG screen performed in Chapter 5 of this thesis, we have therefore found no evidence that acquisition of these genes by NTHi has occurred naturally among our collection of isolates. However, *in vitro* conjugative transfer of ICE*Pmu1* from the *P. multocida* donor to *H. influenzae* Rd KW20 was successful using the conditions described previously at a frequency of 5.73×10^{-6} per recipient. The identity of the transconjugants was confirmed by their PCR identification profiles: isolates were negative for *kmt-1* and positive for P6 and ICE*Pmu1* relaxase and integrase, indicating that the CFUs recovered from the selective plates were *H. influenzae* isolates with ICE*Pmu1* (see Table 7.6).

To further characterise ICE*Pmu1* in our transconjugants, PCR for the detection of specific resistance markers was performed on select transconjugants. Of the AMRGs carried on ICE*Pmu1*, only *erm*(42) was successfully transferred to *H. influenzae* Rd KW20. A number of resistance markers of ICE*Pmu1*, including *msr*(E) and *mph*(E), were not detected in the *H. influenzae* transconjugants, suggesting that ICE*Pmu1* had become truncated during conjugation. Interestingly, all of the genes that were not transferred are located on the same resistance region (region 2) of ICE*Pmu1*, and *tet*(H) was the only resistance marker on this resistance region that was transferred. By contrast, resistance region 1 appeared to be transferred intact (Table 7.6).

Susceptibility testing revealed increases in MICs of a number of antibiotics, including high-level increases of erythromycin, clindamycin and tilmicosin, attributed to the acquisition of *erm*(42) (Table 7.7). A relatively modest increase in MIC of tulathromycin was also

Table 7.6: PCR results for transconjugants.

		Isolate		
	PCR	***P. multocida* (donor)**	***H. influenzae* Rd KW20 (recipient)**	***H. influenzae* Rd KW20 (with ICE*Pmu1*)**
	kmt-1	+	-	-
	P6	-	+	+
	Relaxase	+	-	+
	Integrase	+	-	+
Resistance Region 1	*aphA1*	+	-	+
	sul2	+	-	+
	floR	+	-	+
	erm(42)	+	-	+
	aphA1-strA	+	-	+
	strA-strB	+	-	+
	strA-IS*CR21*	+	-	+
	sul2-strA	+	-	+
	IS*CR21-floR*	+	-	+
	floR-erm(42)	+	-	+
	IS*CR2*-IS*Apl1*	+	-	+
Resistance Region 2	*aadB*	+	-	-
	bla_{oxa-2}	+	-	-
	msr(E)	+	-	-
	aadA25	+	-	-
	mph(E)	+	-	-
	tet(H)	+	-	+
	tetR-IS*26*	+	-	-
	aadB-bla_{oxa-2}	+	-	-
	bla_{oxa-2}-*msr*(E)	+	-	-
	mph(E)-*tet*(H)	+	-	-
	msr(E)-*mph*(E)	+	-	-
	IS*26-tnp*	+	-	-

Table 7.7: MIC results for transconjugants.

	Isolate		
Antibiotic	***P. multocida* (donor)**	***H. influenzae* Rd KW20 (recipient)**	***H. influenzae* Rd KW20 (with ICE*Pmu1*)**
Gentamicin	32	0.5	0.5
Neomycin	≥128	2	≥128
Tetracycline	64	0.5	32
Tilmicosin	≥128	16	≥256
Tulathromycin	≥64	4	32
Chloramphenicol	16	0.5	4
Florfenicol	8	0.25	4
Streptomycin	≥1024	1	256
Ampicillin	0.25	0.12	0.12
Erythromycin	≥64	8	≥64
Clindamycin	≥128	8	≥128
Spectinomycin	256	8	8
Kanamycin	512	4	≥256
Sulfamethoxazole	512	256	≥1024

observed, despite the loss of *msr*(E) and *mph*(E) during conjugation as demonstrated by PCR. Of the antibiotics tested, only gentamicin, spectinomycin and ampicillin MIC did not show notable increases, probably attributable to the loss of the resistance region 2 genes *aadB*, *aadA25* and bla_{OXA-2} during conjugation and further indicating that ICE*Pmu1* had truncated during conjugation to *H. influenzae*.

Based on the PCR and antibiotic susceptibility testing results, we initially speculated that ICE*Pmu1* had undergone a recombination event involving the two copies of *tetR* within resistance region 2 that resulted in the loss of all resistance genes between the *tetR* copies. It is worth noting that *tet*(H), the only resistance marker from resistance region 2 that was retained in the transconjugants and the marker which codes for resistance to our selective agent (tetracycline), is not located between the two copies of *tetR* (see Figure 7.2). This may have allowed for transconjugants with this truncated variant of ICE*Pmu1* to be selected for. We initially tested this hypothesis by running an additional PCR for the presence of the linkage between *tetR* and *tnp*, using the primers ICE_past1-fw and ICE_past2-rv (see Table 7.4) and an annealing temperature of 55°C. Our rationale was that if region 2 was lost through recombination of *tetR*, a single copy of *tetR* should still be present and the use of these primers would result in an amplicon approximately 2900 bp in size. Using *P. multocida* with the complete ICE*Pmu1* as a positive control, this PCR resulted in the production of the expected amplicon, indicating that the *tetR-tnp* linkage was intact and further supporting the above hypothesis.

Attempts to transfer the truncated ICE*Pmu1* to another *H. influenzae* Rd KW20 mutant were successful using the previously described conditions, at a frequency of 1.14×10^{-7} per recipient. This indicates that the transfer functions of ICE*Pmu1* remained active after the initial transfer from *P. multocida* to *H. influenzae*, even in this truncated form. However, attempts to transfer the truncated ICE*Pmu1* to the two NTHi strains included here (*H.*

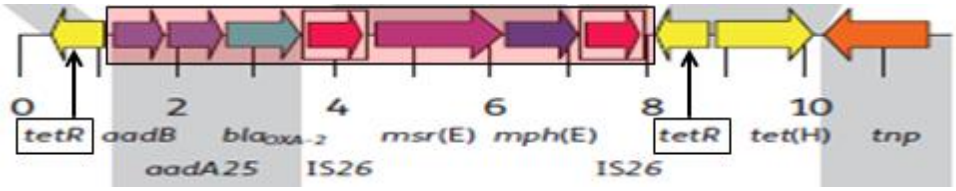

Figure 7.2: Resistance region 2 of ICE*Pmu1* with lost segment indicated. Resistance markers located between the *tetR* copies (highlighted in red) were not transferred to transconjugants generated in this study. The only marker from this resistance region to be transferred successfully, *tet*(H), is located outside of this range.

influenzae NCTC 4560 and Ci6) were not successful under any of the conditions described previously, so transfer of ICE*Pmu1* from *H. influenzae* Rd KW20 to NTHi was not demonstrated in this study. The capacity of ICE*Pmu1* to conjugatively disseminate amongst *H. influenzae* strains if naturally acquired from *P. multocida* remains unclear.

To establish the insertion point of ICE*Pmu1* into the *H. influenzae* genome, we initially used PCR to analyse various $tRNA^{Leu}$ sites to detect any interruptions caused by the insertion of ICE*Pmu1*. However, the amplicons produced by our transconjugants using all 4 sets of $tRNA^{Leu}$ primers (see Section 6.2.6 in Methods) appeared to be identical in size to the amplicon produced by our untransformed *H. influenzae* Rd KW20. This indicated that the $tRNA^{Leu}$ sites were not interrupted by insertion of the ICE, and that ICE*Pmu1* was inserted into a different position. Whole genome sequencing was utilised as an alternative method to establish the insertion point of ICE*Pmu1*. This would also allow for the nature of the truncation event involving resistance region 2 to be confirmed. All six transconjugants that were submitted (3 first generation transconjugants and 3 second generation transconjugants) appeared to be identical after all Contigs were assembled, ordered and aligned against *H. influenzae* Rd KW20 using BRIG (see Figure 7.3). Based on the output given by BRIG, the transconjugants were shown to possess two relatively large segments of additional DNA that *H. influenzae* Rd KW20 lacked (indicated by (a) and (b) in Figure 7.3).

The largest of these segments was revealed to represent ICE*Pmu1*. The integration site was shown to be between nucleotides 1513266 and 1513267 of the *H. influenzae* Rd KW20 genome (based on the numbering system of NC_000907.1 on GenBank). This site represented a $tRNA^{Leu}$ site, listed as HI1424.1 on GenBank (nucleotides 1513211-1513296) and located between HI1424 (a putative integrase/recombinase region) and HI1425 (a fumarate reduction transcriptional regulator). The sequence of this site is demonstrated in

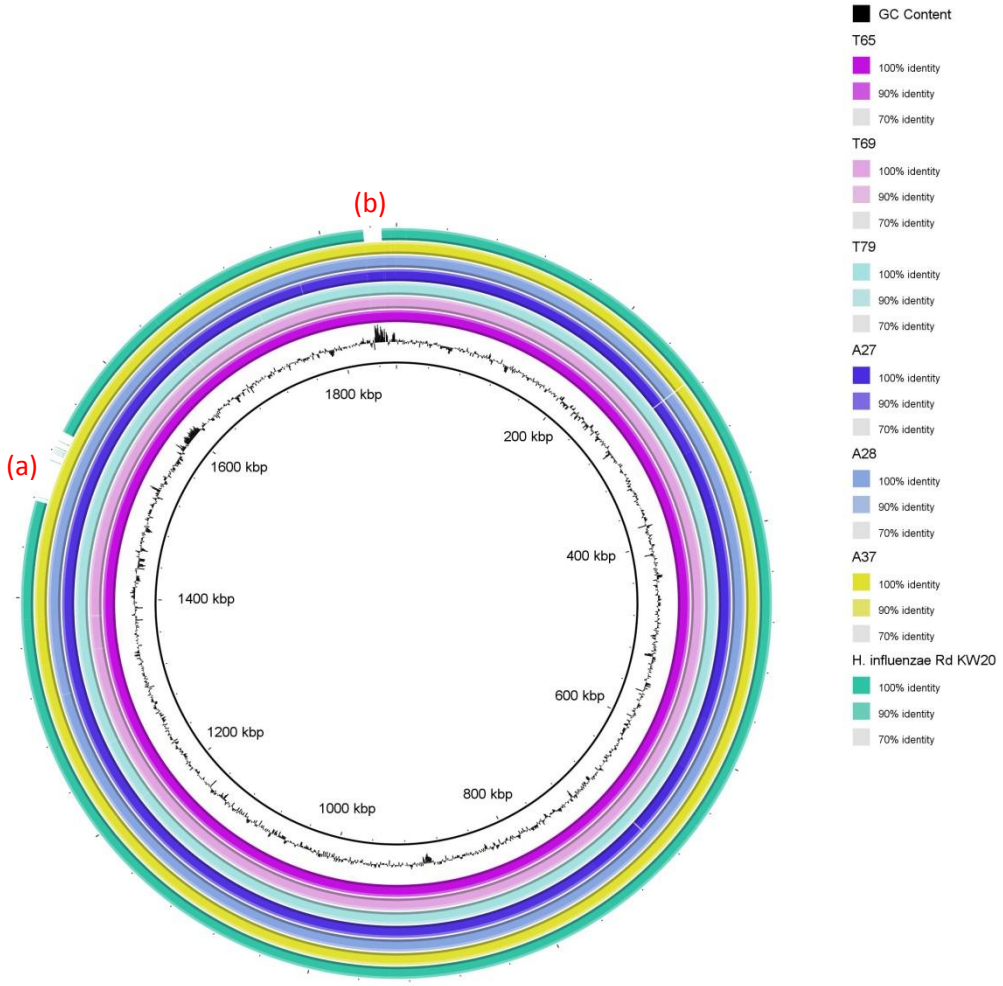

Figure 7.3: Ordered transconjugant sequences aligned against *H. influenzae* Rd KW20 (the outside ring. Two noticeable gaps in *H. influenzae* Rd KW20 (indicated by (a) and (b)) were apparent and indicated the presence of extra DNA segments inserted in those sections of the genome.

Figure 7.4a with the specific integration point indicated. Curiously, this contradicted the previous PCR result that indicated that this site was uninterrupted in the transconjugants; the reasons for this discrepancy remain unresolved but it may be reflective of poor specificity of the primers designed for this study. At the left terminus of ICE*Pmu1* (defined based on the orientation of ICE*Pmu1* within the genome of *H. influenzae* Rd KW20), the previously described[264] flanking sequence 5'-GATTTTGAATCAA-3' was observed (see Figure 7.4b). At the opposite terminus, a similar sequence (5'- GATTTTGAATCCG-3') was found (see Figure 7.4c), although this did not represent a direct repeat of the sequence flanking the left terminus, as is the case in *P. multocida* 36950.[264]

Analysis of ICE*Pmu1* revealed that the non-resistance regions of the ICE were intact. Within resistance region 2, *tnp*, *tet*(H) and a single copy of *tetR* were observed. The second copy of *tetR*, the remaining resistance genes (*aadB*, *aadA25*, bla_{OXA-2}, *msr*(E) and *mph*(E)), and the transposase genes located on region 2 in the native ICE*Pmu1* were not detected within the ICE*Pmu1* of the transconjugants nor within the rest of the genome (see Figure 7.5). This was indicative of a truncation event involving the recombination of the *tetR* copies that resulted in the loss of all DNA situated in between the copies, thus corroborating the findings of the various PCRs and antibiotics resistance tests previously performed as part of this work that had suggested such an event.

Surprisingly, most of resistance region 1 was not detected within the ICE*Pmu1* of the transconjugants. While the first 46 nucleotides of region 1 were detected in its expected position within the ICE, the rest of the region and an 81 nucleotide-long segment that should immediately follow it were missing (see Figure 7.6). Further analysis of the genomes of the transconjugants revealed that these missing components were contained within a separate

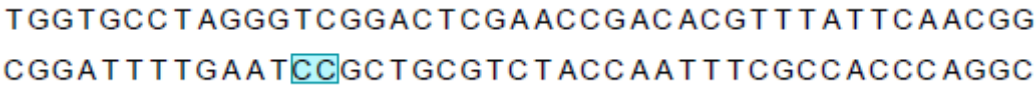

Figure7.4a: tRNA^{Leu} site HI1424.1 of *H. influenzae* Rd KW20. The main ICE structure appeared to be integrated in this site, between the nucleotides highlighted in blue.

Figure 7.4b: Flanking region of the left terminus of the main ICE structure. The flanking sequence 5'-GATTTTGAATCAA-3' was detected here (highlighted in red).

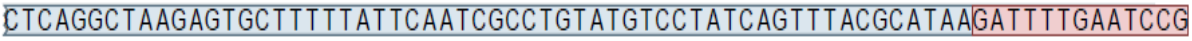

Figure 7.4c: Flanking region of the right terminus of the main ICE structure. The flanking sequence 5'-GATTTTGAATCCG-3' was detected here (highlighted in red). Note that this was not a direct repeat of the flanking sequence of the left terminus but carried.

TGCAAGATTGGCAACCGCAGTGGGCGTTTGGGTTTTTGATGATGTCGTTCATAACAATTCTCCAAATTAAAGGTTGATTAT
CAAAACAAAAACCGTTTTTAAACCCATTAATAAAAAAATGCCATTTAAAACGGTCACTTTTGCACAAGCAACAACCAATGA
TGGTCAATCCTTATTAGCGGTTAATGACTTATTTATTGGCCCTAAAAGCCACACTTCCGCACAGTATATTTTGCAATGGAA
TGGCGCTGAAGAAGTGCAATCTTCATCAGGCATTATTGTATCAACAGGATTGGGATCAACGGGGTGGTTTCAATCTATTCT
TGCTGGTGCGATGGCAATTACAGGAGAAGCTTCGCACCCTCTATTACAAGGCTTTAGCTGGAGTGATCGAAAGCTACAATT
TAGTGTAAGAGAGCCATTTCCAAGTAGAACAACAGGTGTTGCACTGACTTTTGGCACTATTGAGCCTGACTCACCACTGCA
ATTAGGATCTTTGATGCCAGAGAATGGCGTAATTTTCTCTGATGGCATCGAAGATGACTATTTACAATTTAATGCAGGTTG
TATTGCTCACATTGGTATCGCTGACATACAAGGGCAACTAATTAGCCAAAAAGGGCGTCAGCGAATTTAGATTTATTTAGC
GCTCTTTAATACTGTTTCAAGTCCAGAGATCATCACATCAAGGACAAACAAAAATGCAGCATCACCATTATCACTATCCAT
AATTGCAACGGCTTGGGTTAATAATGGCGGATAGGCAACAGTATCCGTCTCTACTTTTTCACGCTCTTTTTGGCTTTCTTG
ATGCTCTTGAGTTTCCAGTACGGAGCCTAATGTAAAATGCGCAATAGAGCTTAATGCATACACGGCTTGAGATAGACTAAA
CCCAGCATCACACAAAAACTGTAGTTGCTGTTCTGATGTCTCAAATTGACTTTCAGAGGGGCGTGTTCCCGCATGAATTTT
GCCACCATCACGATACATTAATAAGGCTTGGCGGAAGCTTTTCGCGTTATTTCGCAAAAAGTCCTGCCATGTTTCATTCGG
CAATGGCAAAACATGATGATGGTGCTTTTGCAAAATAGTTTCTGCTAATGCATCTAACAAAGCGCGTTTATTTTTTACATG
CCAATACAATGTGGGTTGTTCCACACCTATTTTTTGCGCCAGCTTACGCGTTGTTAATCCTTCAATACCAACTTCATTAAG
TAAAATCAACGCATTATCAATAACTTGTTCTTTATCTAGCTTTGCCATTACCTACCTCAAAATAAAATAGCCTTGACAATC
TATCACTGATAGAGATATATTACACCCACTCTATCACTGATAGATTTTTAGGATCTCAATGAATAAATCAATTATTATTAT
ACTGCTGATCACCGTATTAGATGCCATTGGTATCGGGCTTATCATGCCAGTACTCCCTACTCTATTAAATGAATTTGTCAG
TGAAAATTCACTGGCAACCCATTACGGTGTGCTATTAGCGCTCTATGCTACCATGCAGGTTATTTTTGCTCCTATTCTAGG
ACGACTGTCTGATAAATACGGCAGAAAACCCATCTTGCTGTTTTCCCTTTTAGGCGCGGCACTCGACTATCTTTTAATGGC
ATTCTCAACCACACTTTGGATGCTCTATATTGGGCGCATCATTGCGGGGATCACAGGCGCAACAGGTGCCGTATGTGCATC
AGCGATGAGTGATGTGACTCCCGCTAAAAATCGAACTCGCTATTTTGGTTTCTTAGGTGGTGCTTTTGGTGTTGGCCTTAT
TATCGGCCCAATGCTAGGGGGATTATTAGGTGATATCAGTGCTCATATGCCATTTATTTTTGCCGCTATTTCACACTCGAT
ATTATTAATACTCTCTTTGCTCTTTTTCCGAGAAACACAAAAAAGAGAAGCGCTTGTTGCCAATAGGACACCTGAAAACCA
AACTGCCTCAAATACAGTCACTGTTTTTTTTAAGAAAAGCCTCTACTTTTGGTTAGCAACCTATTTTATTATCCAGCTTAT
CGGGCAAATTCCTGCCACCATCTGGGTGCTGTTTACACAATATCGTTTTGATTGGAACACAACTTCTATCGGTATGTCTTT
GGCGGTTCTGGGTGTATTAAATATTTTCTTTCAGGCGATTGTCGCTGGGAAATTGGCACAAAAATGGGGCGAAAAAACCAC
CATTATGATCAGTATGTCTATTGATATGATGGGCTGTTTATTATTAGCGTGGATAGGCCACGTTTGGGTCATCTTACCAGC
ATTAATTTGCTTAGCGGCAGGAGGTATGGGGCAACCCGCATTACAAGGTTATTTATCAAAATCTGTCGATGATAATGCGCA
AGGGAAATTACAAGGTACTCTGGTGAGCCTAACCAATATTACCGGGATCATTGGTCCCCTTTTATTTGCCTTTATTTATAG
TTATAGCGTCGCTTATTGGGATGGTCTGTTATGGCTGATGGGGGCAATACTTTATGCTATGTTGCTTATTACCGCTTATTT
TCACCAAAGAAAAACCACACCTAAAGCTGTTATTTCAACCCCTTAA

Figure 7.5: The truncated resistance region 2 (highlighted in blue).

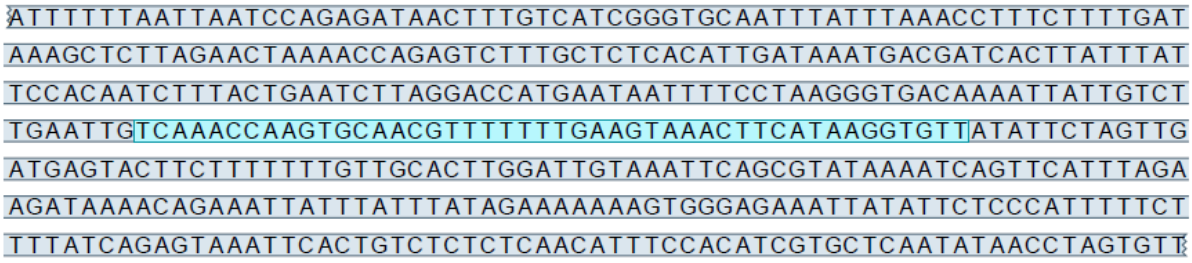

Figure 7.6: Segment of resistance region 1detected in the main ICE structure (highlighted in blue). The complete resistance region 1 appeared to have been integrated in another segment of the H. influenzae Rd KW20 genome.

section of the genome, separated from the main ICE. In all transconjugants, region 1 appeared to be integrated in a site between *metR* (1815309-1816238 of NC_000907.1 on GenBank) and a copy of 16S rRNA (1820456-1822004). This was represented in the ring diagram in Figure 7.3 as the "smaller" gap of the outer ring (*H. influenzae* Rd KW20).

While this "excision" was observed in all transconjugants submitted for sequencing (including the second generation transconjugants), we initially had reservations about whether this separation of region 1 from the ICE was a true event and not simply an erroneous artefact from processing the assembled Contig files. In addition to the event seeming biologically unlikely, such an event has not been reported in other species in which ICE*Pmu1*-carrying transconjugants have been generated. Furthermore, the $tRNA^{Leu}$ site of interest is located among a cluster of RNA-related structures and it is not unreasonable to assume that there is a possibility that region 1 could have been misplaced during the process of ordering the Contig file in Mauve. Notably, a number of features normally located between *metR* and this copy of 16S rRNA in *H. influenzae* Rd KW20 appear to have been displaced, including copies of 5S and 23S rRNA, and tRNA-Ala and tRNA-Ile sites.

Strategies such as PCR mapping of ICE*Pmu1* are being utilised to establish whether the separation of region 1 was merely an artefact of the analysis or a true event; investigations are currently ongoing in this regard. In any case, preliminary investigations of resistance region 1 established that all of the resistance genes that are contained in this region (including *erm*(42) in the native ICE are present in this site, albeit with some apparent rearrangement. The first 1062 nucleotides (the range of which includes the ISApl1 insertion sequence positioned closer to the left terminus in ICE*Pmu1*)[263] were a match against the native ICE*Pmu1*. After this point, the remaining segment of region 1 appeared to have been inverted relative to its original direction in the ICE. A rough diagrammatic representation of

this inversion is shown in Figure 7.7. This resulted in the region no longer being bracketed by IS*Apl1* insertion sequences; instead, the insertion sequences are positioned adjacent to one another. As previously stated, region 1 was located between *metR* and a copy of 16S rRNA in the *H. influenzae* Rd KW20 genome. The left (5'-TTCTTGAATTG-3') flanking region of region 1 in the transconjugants appeared to match the sequence that normally flanks region 1 of the native ICE; the right flanking region was not immediately recognisable, however. Further work is required to confirm the positioning of resistance region 1 in the transconjugants. Inverse PCR would be a useful approach in this situation; by designing primers recognising the edges of region 1 and amplifying the flanking regions, it could be determined whether the region is truly positioned separately from the ICE or if the positioning apparent in the ordered sequence files is due to a processing error.

While there is some unresolved uncertainty about the location of resistance region 1 in the transconjugants, what is clear from the WGS analysis is that the main structure of ICE*Pmu1* integrates into a $tRNA^{Leu}$ site, similar to *P. multocida*, *E. coli* and *M. haemolytica*.[264] This was the case with both first generation and second generation transconjugants. The analysis also confirmed the nature of the recombination event of resistance region 2. Due to financial, legal and quarantine constraints, we were unable to perform further conjugation experiments using *P. multocida* strain 36950, and as such we were unable to attempt to transfer the complete ICE*Pmu1*, including *msr*(E) and *mph*(E), into *H. influenzae* Rd KW20. However, given the nature of the truncation event, there is merit in reattempting these experiments with the replacement of tetracycline with alternate selective agents that may promote the selection of transconjugants with the complete ICE*Pmu1*. The genes *aadB* and *aadA25* both fall within the region that was lost during truncation and code for resistance to gentamicin and spectinomycin, respectively. Therefore, either of these antibiotics may make appropriate agents for selection of transconjugants with the complete ICE*Pmu1*. Ampicillin may also be

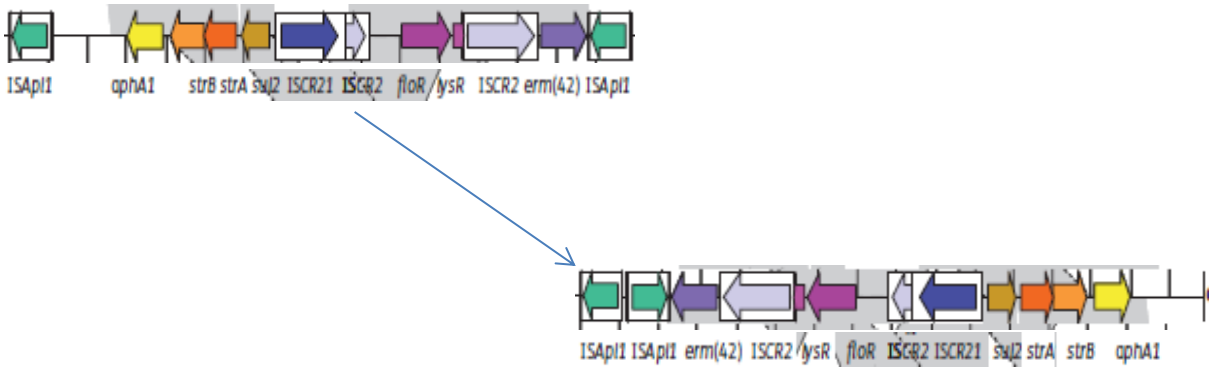

Figure 7.7: Diagram demonstrating the apparent recombination of resistance region 1. The first 1062 nucleotides of the region appeared to be unchanged while the rest of the region was inverted so that the IS*Apl1* copies were adjacent and all genes within the inverted segment were transcribed in the opposite direction.

a possible alternative agent for this purpose due to the presence of bla_{OXA-2} within the lost segment, but given that it has previously been shown that this bla_{OXA-2} does not confer resistance to β-lactam antibiotics in *P. multocida* and *M. haemolytica* hosts,[263] it is uncertain whether this gene will confer resistance to β-lactams to *H. influenzae* and because of this ampicillin may not be an appropriate agent to use in this situation.

The colony counts for the five replicate fitness assays are summarised in Table 7.8. Some between-replicate variation in the counts was apparent. This may be indicative of the occurrence of selection bias during these assays. However, when the average of these counts at each time point was taken, the ratio of cells with ICE*Pmu1* to cells without ICE*Pmu1* was at or close to 1:1 for most time points. When these ratios were plotted (see Figure 7.8), no obvious trend favouring one genotype or the other was apparent and all ratios were within one standard error of 1:1 (represented by an *H. influenzae* (ICE*Pmu1*):*H. influenzae* value of 1 on Figure 7.7), indicating that the acquisition of ICE*Pmu1* did not have an effect on the fitness of *H. influenzae* Rd KW20. In addition, the s values calculated for each replicate and the average of these values (see Table 7.8) did not differ significantly from 0, further supporting the conclusion that ICE*Pmu1* acquisition does not significantly impact fitness. During the stability assays, the number of CFUs that grew on the selective chocolate plates (30 μg/mL kanamycin) remained high across the entire 6 day run; at no point did the cell count fall below 99 CFUs (see Table 7.9). This suggests that ICE*Pmu1* is a relatively stable element in *H. influenzae* and is able to maintain itself even in the absence of selective pressure. Furthermore, the stability results provide further support to the idea that ICE*Pmu1* has no appreciable effect on the fitness of *H. influenzae*. While occasional cells without ICE*Pmu1* were generated during the stability assays, these cells did not dominate the replicates of the fitness or stability experiments as would have been expected if carrying

Table 7.8: Colony counts from the fitness assays.

	Replicate 1			Replicate 2			Replicate 3			Replicate 4			Replicate 5			Average		
Time (h)	**A**	**B**	**A:B**	**A**	**B**	**A:B**	**A**	**B**	**A:B**	**A**	**B**	**A:B**	**A**	**B**	**A:B**	**A**	**B**	**A:B**
0	41	59	0.82	38	62	0.76	73	27	1.46	35	65	0.70	76	24	1.52	53	47	1.05
24	23	77	0.46	9	91	0.18	88	12	1.76	6	94	0.12	53	47	1.06	36	64	0.72
48	24	76	0.48	34	66	0.68	97	3	1.94	8	92	0.16	95	5	1.90	52	48	1.03
72	15	85	0.30	24	76	0.48	93	7	1.86	44	56	0.88	77	23	1.54	51	49	1.01
96	50	50	1.00	16	84	0.32	87	13	1.74	19	81	0.38	77	23	1.54	50	50	1.00
120	85	15	1.70	57	43	1.14	66	34	1.32	8	92	0.16	100	0	2.00	63	37	1.26
144	83	17	1.66	51	49	1.02	33	77	0.66	2	98	0.04	100	0	2.00	54	46	1.08
S	0.0321			0.0197			-0.0239			-0.0209			0.0161			0.0046		

A = No. (*H. influenzae* Rd KW20 CFUs with ICE*Pmu1*), B = No. (*H. influenzae* Rd KW20 CFUs without ICE*Pmu1*)

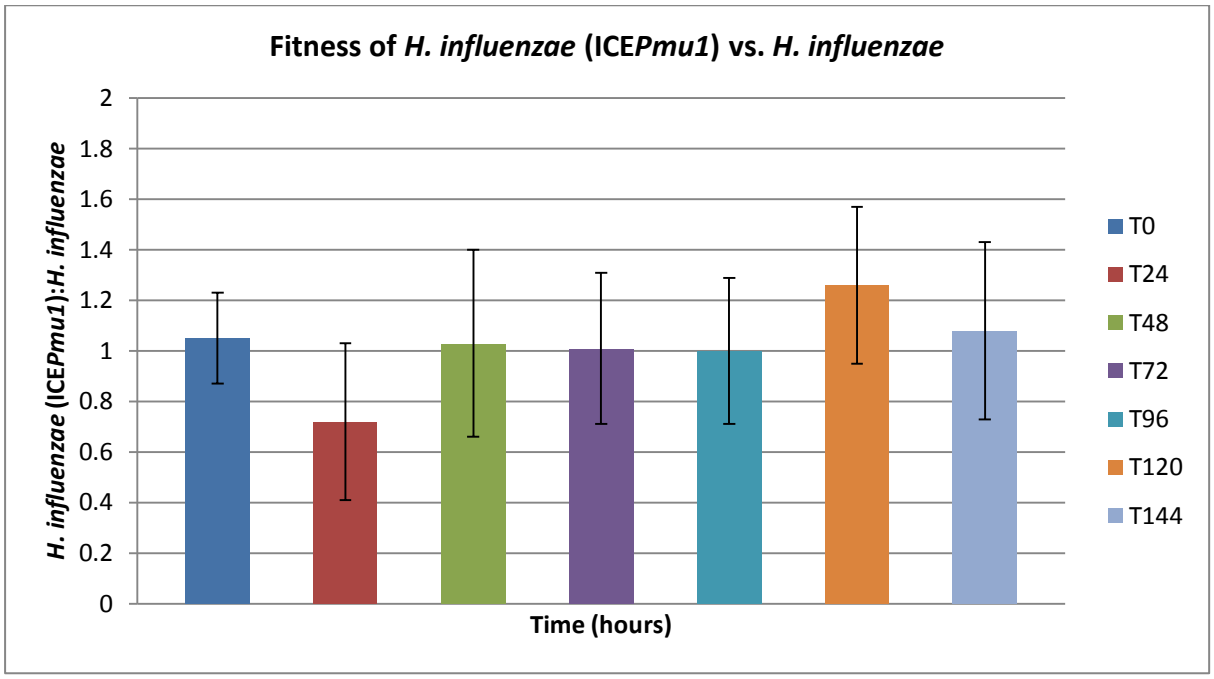

Figure 7.8: Average cell counts from the fitness assays at each 24 hour time point. Cell counts are represented as ratios between the number of cells with ICE*Pmu1* and those without. Note that the error bars represent standard error

Table 7.9: Colony counts from the stability assays.

	Replicate 1			Replicate 2			Replicate 3		
Time (h)	**A**	**B**	**A:B**	**A**	**B**	**A:B**	**A**	**B**	**A:B**
0	100	0	2.00	100	0	2.00	100	0	2.00
24	100	0	2.00	99	1	1.98	99	1	1.98
48	100	0	2.00	100	0	2.00	100	0	2.00
72	100	0	2.00	100	0	2.00	99	1	1.98
96	100	0	2.00	100	0	2.00	100	0	2.00
120	100	0	2.00	99	1	1.98	100	0	2.00
144	99	1	1.98	100	0	2.00	100	0	2.00

A = No. (*H. influenzae* Rd KW20 CFUs with ICE*Pmu1*), B = No. (*H. influenzae* Rd KW20 CFUs without ICE*Pmu1*)

ICE*Pmu1* resulted in a fitness cost for *H. influenzae*. Therefore, we conclude that the acquisition of ICE*Pmu1* has no appreciable cost on fitness of *H. influenzae*.

To summarise the findings of this study, while we have been unable to naturally transfer *msr*(E) and *mph*(E) to *H. influenzae* in this study, we have been able to transfer *erm*(42) and its associated macrolide resistance to *H. influenzae*. Using ICE*Pmu1* as a model, we have demonstrated the potential for AMRG-carrying elements from animal-derived sources such as *P. multocida* to transfer naturally to *H. influenzae*, as well as the potential for *H. influenzae* to acquire macrolide resistance via this mechanism. We have also demonstrated that ICE*Pmu1* (even in the truncated form observed here) is a relatively stable element that has little apparent fitness cost in this species and retains its transfer potential after acquisition by *H. influenzae*, although inter-strain transfer was not observed.

Macrolides and related MLS_B antibiotics remain widely used in animal husbandry worldwide; in Australia specifically, macrolide and streptogramin antibiotics represented approximately 24% of the total animal therapeutic antibiotic market over a 5 year period (2005-2010), with an estimated 54.2 tonnes sold for therapeutic use and 10.7 tonnes sold for growth-promotant use across the period of 2009-2010 alone.[314] While the impact of antibiotic use (particularly for growth promotion) on human health has historically been a controversial issue,[315] links between antibiotic use and resistance in animals have been demonstrated.[315,316] For example, Garcia-Migura et al. (2014) have, in analysing data on the use of antibiotics across nine European countries, previously used linear regression analysis to demonstrate a strong positive correlation between the emergence of resistance among animal flora and the consumption of macrolides in animal husbandry.[317] While we have not found any evidence of the transfer of these particular animal-derived AMRGs among our own collection of isolates (or among the whole genome sequences included in the work of Chapter 5) at this time, it is interesting to speculate about the potential for high-level

macrolide resistance resulting from the acquisition of these genes occurring in and disseminating among clinical isolates of NTHi in the future, especially in light of both the increasing emergence of macrolide resistance in animal-derived *Pasteurellaceae* (potentially resulting from macrolide use in animal husbandry) and previous findings related to the interspecies dissemination of β-lactam resistance involving *Pasteurella* and *Haemophilus*.[262,268]

In considering the likelihood of ICE*Pmu1* and other AMRG-harbouring resistance elements transferring macrolide resistance to NTHi, it is important to consider the frequency of β-lactamase-mediated ampicillin resistance among *Haemophilus* spp. in general. Since the 1970s, β-lactamases in *H. influenzae* have increased in frequency worldwide, and these elements have frequently been carried on genomic islands capable of horizontal transfer, the most well-known of which is ICE*Hin1056* (previously referred to as p1056).[294,295,318] It is known that these elements integrate specifically into $tRNA^{Leu}$,[294,295] much like ICE*Pmu1*. Therefore, in making a judgement regarding the likelihood of ICE*Pmu1* transferring to NTHi in a non-laboratory context, it would be important to establish whether ICE*Pmu1* is able to integrate into $tRNA^{Leu}$ in isolates in which ICE*Hin1056* has already been integrated. If the presence of ICE*Hin1056* inhibited the ability of ICE*Pmu1* to integrate into the *H. influenzae* genome, this would significantly limit the potential for ICE*Pmu1* and related elements to establish themselves in NTHi. Therefore, a potential future study would be to perform the work in this Chapter again using an *H. influenzae* recipient known to carry ICE*Hin1056*.

In any case, we can conclude that transfer events involving AMRG-carrying elements between *Haemophilus* spp. and animal-sourced members of the family *Pasteurellaceae* do not appear to be prevalent at this time and that macrolide resistance through the natural acquisition of animal-derived AMRGs has not been demonstrated in NTHi. However, a potential pathway by which these genes and their associated macrolide resistance phenotype

could be acquired by and disseminated among *H. influenzae* has been identified through this study. Although it is currently unclear how much of a threat such transfer events pose to the susceptibility of NTHi to macrolides, the work presented here may suggest a need to closely monitor the presence of these animal-sourced genes among NTHi isolates.

Chapter 8: General Discussion and Summary

Investigation of the occurrence and effect of acquired macrolide resistance genes (AMRGs) in NTHi is complicated by a number of inter-related and compounding factors. Firstly, there is an extremely large number of different AMRGs (at least 42 *erm* genes, 24 macrolide efflux genes, and 26 macrolide-inactivating enzyme genes have been described),[95] so decisions needed to be made as to whether a study will search for a specific set of these genes, or use an approach that essentially looks for all of those that have currently been described. Secondly, once these genes are detected, determining the effect of these genes on the macrolide susceptibility phenotype is complicated by the presence of complex regulatory regions in some genes that may limit their expression in the genetic environment of the new host. The effect on phenotype of AMRGs is further complicated by the possible presence of a range of chromosomal mutations that may also contribute to resistance in a given AMRG-carrying isolate. The effect of specific AMRGs on phenotype is perhaps best determined in a controlled genetic background, either by artificially introducing the AMRG on its native replicon, or by cloning into a suitable vector. Again, these approaches are complicated by inefficient conjugative transfer of AMRGs into *H. influenzae* from many of the organisms in which AMRGs are prevalent, and by both the difficulty in sourcing appropriate vectors and the difficulty in transforming many strains of *H. influenzae*. These difficulties have been encountered and dealt with in various ways throughout the work of this thesis.

The work of Chapter 4 was primarily focused on the AMRGs *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*(A) and *mef*(E). It is important to stress that these AMRGs were not selected specifically because they are commonly encountered among particular species in which genetic exchange with *H. influenzae* occurs readily. These genes were selected because, in addition to being frequently encountered among a wide variety of species, they were recently

reported in high prevalence among NTHi isolates derived from a specific CF patient group.[93] Our search for these genes in 186 clinical respiratory isolates of NTHi, from both CF and non-CF patients, did not uncover any isolates with any of these genes. Given the previous report of Roberts et al. (2011),[93] this finding was somewhat surprising despite variations in the patient groups from which the isolates of both studies were derived. In any case, our failure to detect any isolates with at least one AMRG limited our options to further investigate the role these genes play in reducing macrolide susceptibility in NTHi, and as it was not possible to source any such isolates from the authors of the only study in which these genes were detected in large numbers, alternative approaches were required.

One of the options was to attempt to transfer these genes into *H. influenzae* by conjugative transfer *in vitro*, described in Chapter 6 of this thesis. However, these attempts did not produce any AMRG-carrying *H. influenzae* transconjugants. This finding was not surprising, and the fact that transfer does not occur even in optimised laboratory conditions perhaps reflects the rarity of these genes in NTHi compared to the prevalence of these genes in other species that frequently occupy the upper respiratory tract. This is further supported by our inability to detect AMRGs among any of the NTHi isolates examined in Chapter 4, or among the 89 publicly available whole genome sequences (WGSs) interrogated in Chapter 5.

Given the inability to transfer these genes by conjugation, they were artificially cloned into a shuttle vector, pLS88, and then transformed into *H. influenzae* Rd KW20. This vector is a naturally occurring plasmid that has not been further manipulated to allow for additional useful functions such as controlling the level of expression. The use of a dedicated expression vector may have provided additional useful data, but these are not readily available for *H. influenzae*, and modification of our existing vector was beyond the scope of this study. The cloning of the AMRGs into pLS88, with consistent expression, was useful as it allowed us to confirm expression of these genes and correlate this expression to a phenotype. This had the

advantage of demonstrating the effect of these genes in isolation of other underlying mechanisms such as chromosomal mutations. A useful future extension of this current work, would be to examine the effect of various AMRGs in unison with other specific mechanisms, including specific L4, L22 and 23S rRNA mutations, or overexpressed *acrAB* due to alterations in *acrR*. The relevance of this approach is demonstrated by the previous description of *H. parainfluenzae* with both a *mef*(A) and L4 substitution, where the respective contributions of each mechanism to the high level resistance phenotype was not determined.[257] These types of studies will be challenging in *H. influenzae* in the absence of optimised systems in which to create specific alterations of these genes, although in future studies this may be possible through the use of a potentially suitable suicide vector for unmarked allelic exchange.[319] This approach would be useful to examine the degree to which L4 and L22 mutations affect the resistance phenotype observed with various AMRGs because the specific mutations in L4 and L22 associated with resistance are well known. This approach may not yet be feasible for the *acrR* mutations, as this area is relatively new in *H. influenzae* and further work would be required to establish what specific mutations should be introduced. Additional difficulties would be encountered for examining the effect of 23S rRNA mutations in this context. This is because the number of mutated copies required to produce reduced susceptibility has not been determined, and controlling the number of mutant copies generated in a mutagenesis experiment would be challenging.

In addition to the rationale provided by the findings of Roberts et al.,[93] the initial selection of the AMRGs described above was made based on the likelihood of an encounter between NTHi and other organisms in which these genes are highly prevalent. However, another approach is to look at AMRGs found in organisms that are more closely related to *H. influenzae* phylogenetically. This is especially useful for organisms in which there is a precedent for transfer of antibiotic resistance determinants to *H. influenzae*, as is the case for

P. multocida.[268] ICE*Pmu1*, a mobile element detected in a bovine-derived *P. multocida* isolate and that has had its complete structure elucidated,[263,264] is a carrier of the AMRGs *erm*(42), *msr*(E) and *mph*(E). As a result of a presentation given by myself on behalf of our group at the Australian Society for Microbiology Annual Scientific Meeting in Canberra (2015) on the work described in Chapter 4 of this thesis and a subsequent encounter with Dr. Stefan Schwarz (Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany) after this presentation, I was given a unique opportunity to travel to Dr. Schwarz's laboratory in Germany and perform conjugation experiments involving the transfer of ICE*Pmu1* from *P. multocida* to *H. influenzae*. While it was possible to conjugatively transfer ICE*Pmu1*, a recombination event occurred during the conjugation that produced a truncation of the ICE. This truncation resulted in the loss of the *msr*(E) and *mph*(E), although the *erm*(42) was retained and did produce a macrolide resistant phenotype. The ICE was additionally shown to be stable in *H. influenzae* and maintained its transfer functions while having little apparent effect on the fitness of the host organism. This provides some evidence for an alternative potential pathway in which AMRGs could be acquired by NTHi.

Chapter 5 represents a preliminary examination of the use of whole genome sequencing (WGS) to screen for AMRGs in our clinical isolates with high level macrolide resistance. The panel for this screen included the same AMRGs investigated in Chapter 4, where these genes were absent from the clinical isolates screened; the failure to detect any of these AMRGs in these WGSs supported the findings of Chapter 4. The advantage of using WGS for this purpose was that it was possible to screen for a very large set of genes that would have been impractical with conventional PCR. Although we did not detect any AMRGs, an additional advantage of this approach was that we could also look for the presence of

established chromosomal mutations that may have been contributing to the resistant phenotype.

The research findings presented in this thesis suggest that acquisition and subsequent expression of *erm*(A), *erm*(B) and *erm*(C) would produce a macrolide resistant phenotype in *H. influenzae* in the absence of any contributory mutations in macrolide-relevant chromosomal genes. However, AMRGs do not appear to be widespread among wild-type isolates of NTHi and the high prevalence of AMRGs described in the Roberts et al.[93] study seems to be unique to the circumstances of that particular study. At present, high-level macrolide resistance in NTHi can primarily be attributed to chromosomal mutations, including in the L4 and L22 genes, and in 23S rRNA. To reconcile these observations and potentially foresee how prevalence of these genes in NTHi could shift in the future, it is important to consider issues relating to the mechanisms through which these genes could be acquired by and disseminated in NTHi. The variety and high prevalence of AMRGs in Gram positive bacteria of the respiratory tract attests to the presence of sufficient selective pressure to render the acquisition and maintenance of these genes attractive in an evolutionary sense. Whether the inherent intrinsic low susceptibility of *H. influenzae* to macrolides presents the same advantages for the acquisition and maintenance of these genes is difficult to assess, but the presence of naturally occurring isolates with high level macrolide resistance due to mutations in ribosomal elements suggests that this is a distinct possibility. Despite this, there are two different but plausible pathways by which *Haemophilus* spp. might acquire AMRGs. One possibility is acquisition from Gram positive organisms in which AMRGs are highly prevalent. Previous reports suggest that there is the general flux of resistance genes that favours movement from Gram positive organisms to Gram negative organisms.[320] In the case of AMRGs, acquisition from Gram positive organisms is favoured by both the high prevalence of these genes in Gram positive respiratory organisms and the frequency of

interaction between these organisms and *Haemophilus* spp. afforded by their co-habitation in the respiratory tract.[321] As previously discussed in this thesis, many of these AMRGs are present on conjugative elements in Gram positive organisms, and conjugation is a mechanism of genetic transfer of very broad host range.[321,322] However, while resistance determinants from Gram positives are readily expressed in Gram negatives, a barrier is presented by the frequent inability of the conjugative elements to replicate well in the new Gram negative host.[320,321] However, if the genes are on transposable elements, then conjugation followed by transposition of the AMRGs either to the recipient genome or co-resident native extra-chromosomal genetic elements is a plausible mechanism for the transfer.[321] This proposed mechanism is supported by the observation that many AMRGs in Gram positive organisms are on transposable elements and that *Haemophilus* spp. frequently carry plasmids or ICEs that could act as vectors for these transposable elements. The other possibility, which we have briefly examined in this thesis, is that *Haemophilus* spp. might acquire AMRGs from less phylogenetically distant organisms such as animal-derived organisms of the family *Pasteurellaceae*. While the prevalence of AMRGs within organisms of this family may be high in some situations, the barrier here is the infrequency with which human isolates of NTHi would encounter these organisms in a manner conducive to conjugation, although as previously discussed, there is precedent for this to occur.[262,268]

While we conclude that there is currently little evidence of the widespread emergence of AMRGs in NTHi, recent developments in this area have reinforced the need to monitor for AMRGs in NTHi. Since the commencement of this thesis, there has been at least one other report of an AMRG in a clinical isolate of NTHi which must be addressed here. Seyama et al. (2016) have reported the presence of *mef*(A) in a paediatric respiratory isolate of NTHi exhibiting high-level macrolide resistance (azithromycin MIC = 64 µg/mL; clarithromycin MIC = 128 µg/mL).[254] This isolate was not found to carrying known macrolide resistance-

associated mutations in L4, L22 or 23S rRNA, and did not exhibit overexpression of *acrB*, leading the authors of that study to conclude that *mef*(A) was likely producing the macrolide resistance phenotype of the isolate. Further analysis of the isolate revealed that *tet*(M) was located close by to *mef*(A), and that the 6445bp region from *tet*(M) to *mef*(A) was identical to a segment of the streptococcal transposon Tn*916*, suggesting that these genes may have been acquired from a streptococcal species. However, the authors were not able to demonstrate conjugative transfer of *mef*(A) to *H. influenzae* Rd KW20 or to clinical isolates of *H. influenzae* or *S. pneumoniae*. No mention of *msr*(D) is made throughout the report, but we performed a BLAST search of CDS_5 of the 6445bp region deposited on GenBank (GenBank accession LC168847) which indicates that *msr*(D) is present on the element adjacent to *mef*(A), and as such its role in the exhibited phenotype should not be ruled out. Regardless, this very recent study further demonstrates that AMRG acquisition by NTHi can occur naturally, and we believe that this evidence, in conjunction with other issues such as the increasing use of macrolides in both animals and humans, the prevalence of AMRGs in other organisms, and the numerous potential pathways through which AMRG transfer could occur, suggest that the emergence of AMRGs may be an imminent threat and that it is prudent to undertake ongoing periodic surveillance for these genes in NTHi.

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Appendix 2: Supplementary data from analyses performed in Chapter 5

Table A2.1: List of polymorphisms in CA1.

Pos.*	Gene	Ref	CA1	Pos.*	Gene	Ref	CA1	Pos.*	Gene	Ref	CA1	Pos.*	Gene	Ref	CA1
878699	*tpx*	C	T	886944	*gpmA*	A	G	893061	*nadR*	G	T	898547	*ftsX*	G	A
878815	*tpx*	A	C	887034	*gpmA*	T	C	893151	*ribB*	G	A	898563	*ftsX*	G	C
878917		C	G	887058	*gpmA*	A	G	893259	*ribB*	A	C	898598	*ftsX*	T	C
878954		T	G	887190	*gpmA*	C	T	893268	*ribB*	C	T	898625	*ftsX*	A	G
879065		G	A	887310	*gpmA*	C	T	893292	*ribB*	T	C	898634	*ftsX*	C	T
879067		A	G	887373	*gpmA*	A	G	893304	*ribB*	T	C	898688	*ftsX*	G	A
879419	*purL*	T	G	887484	*gpmA*	T	A	893311	*ribB*	C	A	899005	*atoB*	A	G
879458	*purL*	C	T	887643	*rpmE*	G	A	893318	*ribB*	C	A	899092	*atoB*	C	T
879491	*purL*	T	C	887703	*rpmE*	G	A	893392	*ribB*	T	C	899373	*atoB*	C	T
879563	*purL*	A	G	887715	*rpmE*	A	G	893433	*ribB*	A	C	899389	*atoB*	A	G
880948	*purL*	G	A	887751	*rpmE*	C	A	893445	*ribB*	C	T	900252	*atoE*	C	T
881180	*purL*	G	A	887780	*rpmE*	C	T	895833	NTHI0928	T	C	900260	*atoE*	T	C
881240	*purL*	A	G	887847	*rpmE*	G	A	896003	*ftsY*	A	G	900408	*atoE*	T	G
881262	*purL*	T	C	887857	*rpmE*	C	T	896048	*ftsY*	A	G	900474	*atoE*	C	T
881277	*purL*	G	T	887927	*mutY*	G	A	896103	*ftsY*	A	G	900489	*atoE*	T	A
881303	*purL*	T	C	887988	*mutY*	T	C	896106	*ftsY*	C	A	900497	*atoE*	T	G
881508	*purL*	A	G	888004	*mutY*	T	C	896186	*ftsY*	G	T	900534	*atoE*	C	T
881528	*purL*	C	T	888016	*mutY*	G	A	896198	*ftsY*	G	T	900603	*atoE*	G	A
881546	*purL*	G	C	888061	*mutY*	C	A	896264	*ftsY*	G	A	900618	*atoE*	C	T
881603	*purL*	C	T	888151	*mutY*	T	C	896372	*ftsY*	G	T	900660	*atoE*	T	A
881642	*purL*	G	A	888238	*mutY*	A	G	896375	*ftsY*	G	A	900669	*atoE*	G	A
881679	*purL*	C	T	888280	*mutY*	C	T	896412	*ftsY*	G	A	901691	*atoA*	A	C
881696	*purL*	T	C	888286	*mutY*	A	G	896438	*ftsY*	T	C	901853	*atoA*	G	A
882050	*purL*	T	C	888292	*mutY*	A	G	896669	*ftsY*	C	T	902211	*atoD*	T	C
882197	*purL*	C	T	888319	*mutY*	G	A	896678	*ftsY*	T	C	902220	*atoD*	T	G
882212	*purL*	A	T	888343	*mutY*	G	A	896822	*ftsY*	G	A	902325	*atoD*	T	C
882319	*purL*	G	A	888376	*mutY*	C	T	896894	*ftsY*	C	T	902391	*atoD*	T	A
883052	*purL*	T	C	888622	*mutY*	T	A	896903	*ftsY*	A	G	902565	*atoD*	C	G
883300		G	C	888644	*mutY*	A	G	896936	*ftsY*	C	T	902848	*atoD*	A	G
883302		T	C	888756	*mutY*	A	G	896966	*ftsY*	A	G	902849	*atoD*	A	G
883325		G	A	888798	*mutY*	A	C	897181	*ftsE*	A	G	902871	NTHI0936	A	G
883353		T	G	888806	*mutY*	T	G	897236	*ftsE*	A	C	902907	NTHI0936	C	T
883920	*lex2B*	A	C	888819	*mutY*	A	G	897239	*ftsE*	C	G	902968	NTHI0936	G	A
883970	*lex2B*	G	A	888860	*mutY*	C	T	897338	*ftsE*	G	A	902992	NTHI0936	G	A
884917	NTHI0914	G	C	889160	NTHI0920	T	C	897666	*ftsE*	C	T	903114	NTHI0936	G	A
884938	NTHI0914	C	T	889172	NTHI0920	T	A	897668	*ftsE*	A	G	903372	NTHI0936	T	C
885121	NTHI0914	T	C	889221	NTHI0920	G	A	897695	*ftsE*	C	T	903534	NTHI0936	C	T
885298	NTHI0914	T	C	889223	NTHI0920	T	C	897764	*ftsE*	C	T	903840	NTHI0936	T	C
885443	NTHI0915	A	G	889286	NTHI0920	G	A	898046	*ftsX*	C	A	903849	NTHI0936	C	T
885576	NTHI0915	G	C	889355	NTHI0920	T	C	898109	*ftsX*	A	G	903891	NTHI0936	G	A
885596	NTHI0915	A	G	889381	*mltC*	C	T	898118	*ftsX*	C	T	904767	*rplC*	C	T
885678	NTHI0915	A	T	889873	*mltC*	A	T	898133	*ftsX*	A	G	905732	*rplW*	T	C
885852	NTHI0915	T	C	889909	*mltC*	T	C	898199	*ftsX*	C	T	905747	*rplW*	G	A
885870	NTHI0915	T	C	889913	*mltC*	G	A	898244	*ftsX*	G	T	905750	*rplW*	C	T
886193	NTHI0915	T	C	889939	*mltC*	A	G	898247	*ftsX*	A	G	906220	*rplB*	A	C
886265	NTHI0915	G	T	889948	*mltC*	A	G	898273	*ftsX*	T	C	906247	*rplB*	T	C
886331	NTHI0915	T	C	892805	*nadR*	G	A	898292	*ftsX*	G	A	907812	*rpsC*	T	C
886376	NTHI0915	G	A	892811	*nadR*	A	G	898469	*ftsX*	C	T	1006144	NTHI1058	C	G
886428	NTHI0915	T	G	893034	*nadR*	G	A	898478	*ftsX*	C	A	1849392	*nth*	A	C
886521	NTHI0915	T	A	893057	*nadR*	C	T	898520	*ftsX*	G	A				

*Position numbering based on that of NC_007146

Table A2.2a: List of polymorphisms in CA4 and CA6, part 1.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
3808	HI0003	T	C	527908	HI0511	A	T	586935	*gyrB*	A	T	592499	*oxyR*	C	A
45496	HI0043	G	A	529704	hindIIM	G	T	587220	*gyrB*	C	T	592517	*oxyR*	G	A
46301	HI0043	A	T	551578	*tyrP*	T	A	587223	*gyrB*	G	T	592568	*oxyR*	A	G
47284		A	T	558150		G	A	587271	*gyrB*	C	T	592974	HI0572	C	T
52337	HI0051	G	A	559856	*aspA*	G	T	587334	*gyrB*	A	G	593008	HI0572	T	A
52396	HI0051	G	A	566055	*groEL*	C	A	587412	*gyrB*	A	G	593085	HI0572	C	T
54392	HI0053	C	A	583301	*asnA*	A	C	587646	*gyrB*	G	T	593124	HI0572	C	T
55727	*uxuA*	G	A	583385	*asnA*	G	A	587649	*gyrB*	C	T	593934	*fkpA*	A	G
61735	*msbA*	A	C	583625	*asnA*	T	A	587682	*gyrB*	G	A	593936	*fkpA*	A	G
68938	HI0066	C	T	583628	*asnA*	C	T	587706	*gyrB*	A	C	594022	*fkpA*	C	T
77720	*recN*	G	A	583670	*asnA*	C	T	587712	*gyrB*	T	C	594220	*fkpA*	T	C
84550		C	T	583676	*asnA*	T	C	587733	*gyrB*	A	T	594310	*fkpA*	C	G
107030	*hitC*	T	A	584093	*gph*	C	G	587736	*gyrB*	A	G	594406	*fkpA*	A	G
108089		G	T	584094	*gph*	G	T	587961	*gyrB*	C	T	594553	*fkpA*	G	A
117334	HI0109	C	T	584146	*gph*	C	T	588165	*tex*	T	C	594794	HI0575	C	G
153327	*rnhA*	C	A	584155	*gph*	A	G	588169	*tex*	A	G	594848	HI0575	G	A
153443	*rnhA*	C	A	584652	*gph*	G	A	588205	*tex*	A	G	594866	HI0575	C	T
161008	HI0145	G	A	584671	*gph*	A	G	588211	*tex*	C	G	594869	HI0575	C	T
161069	HI0145	C	G	584842	*dod*	G	A	588224	*tex*	T	C	594971	HI0575	T	C
161070	HI0145	G	C	584851	*dod*	T	A	588225	*tex*	G	T	594974	HI0575	C	T
183645	HI0170	C	A	584857	*dod*	C	T	588246	*tex*	G	A	595013	HI0575	T	C
185412		C	A	584893	*dod*	G	T	588249	*tex*	A	G	595016	HI0575	T	C
186728	HI0173	A	T	585001	*dod*	A	G	588402	*tex*	C	T	595066	HI0575	T	C
186732	HI0173	T	A	585040	*dod*	C	T	588612	*tex*	C	T	595070	HI0575	G	T
205057	*fur*	G	C	585046	*dod*	A	G	588654	*tex*	G	T	595205	HI0575	C	T
205178	*fur*	C	T	585068	*dod*	A	G	588662	*tex*	T	C	595265	HI0575	T	A
205195	*fur*	C	T	585076	*dod*	A	G	588684	*tex*	C	T	595271	HI0575	T	C
209262	HI0195.1	T	A	585178	*dod*	C	T	588717	*tex*	A	G	595375	HI0576	G	A
224199	*dam*	A	T	585202	*dod*	G	A	588804	*tex*	T	A	595408	HI0576	A	T
240237	HI0219a	G	A	585208	*dod*	G	A	590489	*tex*	A	C	595409	HI0576	T	G
240329	HI0219a	T	C	585235	*dod*	T	A	590596	*greB*	G	A	595425	HI0576	A	G
241737	HI0220.2	A	T	585268	*dod*	C	T	590647	*greB*	G	A	595453	HI0576	T	C
241739	HI0220.2	A	T	585277	*dod*	C	T	590692	*greB*	T	C	595498	HI0576	C	T
255835	*brnQ*	G	T	585491	*dod*	C	T	590695	*greB*	T	C	595550	HI0576	G	A
283562	HI0251	C	T	585534		T	A	590740	*greB*	A	G	595702	HI0576	G	A
283574	HI0251	C	T	585585	*gyrB*	C	T	590857	*greB*	T	C	595716	HI0576.1	A	G
286431	dapA	G	A	585684	*gyrB*	T	C	590959	*greB*	C	A	596051	HI0576.1	G	A
291927	HI0261	G	A	585765	*gyrB*	C	T	591135	HI0570	T	C	596123	HI0577	A	C
292028		G	A	585789	*gyrB*	A	G	591150	HI0570	T	A	596129	HI0577	T	C
341460	HI0308	C	T	585807	*gyrB*	C	T	591168	HI0570	T	C	596581	*tuf*	A	G
351674	HI0322	T	A	585812	*gyrB*	A	G	591195	HI0570	A	C	596620	*tuf*	G	C
375590	*napC*	C	A	585924	*gyrB*	T	C	591368	HI0570	A	G	596656	*tuf*	G	A
390021	HI0366	G	A	586041	*gyrB*	C	T	591639	HI0570	T	C	596659	*tuf*	T	A
401469	HI0380	G	A	586062	*gyrB*	A	T	591654	HI0570	G	T	596782	*tuf*	T	C
402013		C	T	586110	*gyrB*	C	A	591660	HI0570	A	C	597232	*tuf*	G	T
412858	*rnd*	G	A	586195	*gyrB*	T	C	591708	*oxyR*	T	C	597265	*tuf*	G	A
453173	*glmS*	C	G	586200	*gyrB*	A	G	592451	*oxyR*	A	G	597298	*tuf*	A	T
453174	*glmS*	G	C	586452	*gyrB*	G	C	592454	*oxyR*	T	C	597875	*fusA*	C	T
474778	HI0452	G	C	586809	*gyrB*	G	A	592463	*oxyR*	A	G	597878	*fusA*	A	T
474826	HI0452	G	A	586902	*gyrB*	C	T	592487	*oxyR*	C	T	597935	*fusA*	A	T

*Position numbering based on that of NC_000907

Table A2.2b: List of polymorphisms in CA4 and CA6, part 2.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
598268	*fusA*	G	A	827415	*nadR*	G	A	832318	*ftsE*	T	C	837482		A	G
598300	*fusA*	C	G	827421	*nadR*	A	G	832319	*ftsE*	T	G	837496		G	A
598352	*fusA*	T	G	827644	*nadR*	G	A	832357	*ftsE*	C	A	837518	HI0775	C	T
598370	*fusA*	A	G	827667	*nadR*	C	T	832534	*ftsX*	G	A	837579	HI0775	G	A
598412	*fusA*	G	A	827671	*nadR*	G	T	832705	*ftsX*	A	G	837603	HI0775	G	A
598649	*fusA*	T	C	827761	*ribB*	G	A	832762	*ftsX*	C	T	837725	HI0775	G	A
598772	*fusA*	G	A	827780	*ribB*	T	C	832783	*ftsX*	A	G	838699	*rpsJ*	C	T
599959	*rpS7*	G	A	827869	*ribB*	A	C	832789	*ftsX*	C	T	839851	*rplD*	A	G
599974	*rpS7*	G	A	827878	*ribB*	C	T	832795	*ftsX*	T	C	840223	*rplD*	C	A
599983	*rpS7*	G	A	827902	*ribB*	T	C	832858	*ftsX*	A	G	840256	*rplD*	G	A
600040	*rpS7*	C	T	827917	*ribB*	T	C	832884	*ftsX*	T	C	840271	*rplD*	G	A
600199	*rpS7*	A	G	827928	*ribB*	C	A	832885	*ftsX*	T	G	840830	*rplB*	A	C
600661	*rpsL*	C	T	827951	*ribB*	G	G	832900	*ftsX*	C	T	840857	*rplB*	T	C
600718	*rpsL*	A	G	828040	*ribB*	C	T	832909	*ftsX*	A	T	840929	*rplB*	C	T
600793	*rpsL*	A	T	828043	*ribB*	T	C	832975	*ftsX*	G	A	842032	*rplV*	G	C
600806	*rpsL*	T	C	828055	*ribB*	C	T	833517	*atoB*	C	A	842265	*rpsC*	G	A
601538	*gidA*	C	A	828061	*ribB*	T	C	833984	*atoB*	C	T	845361	*rplX*	C	G
601544	*gidA*	A	C	830360	HI0767	C	T	834000	*atoB*	A	G	845396	*rplX*	C	T
601560	*gidA*	C	T	830378	HI0767	G	T	834042	*atoB*	A	T	845489	*rplX*	A	G
601568	*gidA*	A	G	830390	HI0767	A	T	834219	*atoB*	G	A	845995	*rplE*	A	G
601625	*gidA*	C	A	830395	HI0767	T	C	834627	*atoB*	G	A	846034	*rplE*	T	C
601642	*gidA*	A	G	830426	HI0767	A	C	834743	*atoE*	A	G	846109	*rplE*	T	C
601673	*gidA*	T	C	830429	HI0767	T	A	834845	*atoE*	C	T	846921	*rpsH*	C	T
601706	*gidA*	T	A	830821	*ftsY*	T	A	834938	*atoE*	A	C	847747	*rplR*	T	A
601748	*gidA*	T	C	830845	*ftsY*	A	G	835085	*atoE*	C	T	847756	*rplR*	A	C
601772	*gidA*	G	A	830924	*ftsY*	G	A	835100	*atoE*	T	A	847837	*rplR*	A	T
608507	*pepE*	C	T	831001	*ftsY*	T	C	835145	*atoE*	C	T	848301	*rpsE*	A	G
615544		T	A	831043	*ftsY*	C	T	835214	*atoE*	G	A	848722	*rplO*	G	A
615545		A	G	831052	*ftsY*	C	T	835229	*atoE*	C	T	850015	*secY*	C	T
622425	*recA*	A	T	831130	*ftsY*	A	G	835271	*atoE*	T	A	850345	*secY*	A	G
623483	*tfoX*	G	T	831137	*ftsY*	G	A	835280	*atoE*	G	A	850484	*rpmJ*	C	T
633274	*cyaA*	C	T	831202	*ftsY*	A	G	835510	*atoE*	T	C	851073	*rpS11*	C	G
639329	HI0608	C	T	831262	*ftsY*	A	T	835646	*atoE*	C	T	851074	*rpS11*	G	C
641342		C	T	831433	*ftsY*	G	A	836034	*atoA*	C	A	853414	*rplQ*	T	C
677077	HI0635	T	A	831547	*ftsY*	C	T	836440	*atoA*	A	G	853730		G	A
677143	HI0635	C	T	831607	*ftsY*	A	G	836463	*atoA*	A	C	853743		C	T
677144	HI0635	G	T	831643	*ftsY*	C	T	836464	*atoA*	G	A	854380		T	A
677154	HI0635	T	C	831835	*ftsE*	C	T	836522	*atoA*	T	G	854763		T	C
677157	HI0635	T	C	831841	*ftsE*	A	G	836822	HI0774	T	C	854772		T	C
678411	HI0636	C	T	831850	*ftsE*	C	G	836831	HI0774	T	G	858995	*pckA*	G	A
682239	*rplJ*	C	G	831877	*ftsE*	T	A	836846	HI0774	C	T	865937	*alaS*	G	C
682240	*rplJ*	G	C	831964	*ftsE*	T	C	836946	HI0774	C	T	880989	HI0829	G	A
705310	*oapA*	G	C	832009	*ftsE*	G	A	837002	HI0774	T	A	885555	*frdA*	C	G
725490		C	A	832054	*ftsE*	C	T	837026	HI0774	G	A	885556	*frdA*	G	C
737510	*hel*	G	A	832084	*ftsE*	T	A	837029	HI0774	G	A	885762	*frdA*	C	T
745159	HI0698	G	A	832087	*ftsE*	A	G	837059	HI0774	T	A	885810	*genX*	G	T
753312	*mutS*	T	G	832159	*ftsE*	A	G	837137	HI0774	G	T	885944	*genX*	A	T
760350	HI0712	A	G	832222	*ftsE*	A	G	837441	HI0774	C	T	899386	HI0852	C	A
760386	HI0712	A	G	832270	*ftsE*	A	G	837459	HI0774	A	G	907024	*clpB*	C	G
765944	*nusG*	G	C	832306	*ftsE*	C	T	837460	HI0774	A	G	907025	*clpB*	G	C

*Position numbering based on that of NC_000907

Table A2.2c: List of polymorphisms in CA4 and CA6, part 3.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
924509	*wbaP*	C	T	1016913	*rumB*	C	T	1025443	HI0967	T	C	1032320		T	C
931827	HI0878	G	A	1016994	*exoII*	G	A	1025444	HI0967	G	A	1032344		T	C
932121	*rpmA*	A	C	1016999	*exoII*	A	G	1025687	*menB*	T	A	1032362		C	T
932122	*rpmA*	C	T	1017134	*exoII*	G	C	1025708	*menB*	T	C	1032647		C	A
932799		T	A	1017369	*exoII*	A	G	1025732	*menB*	G	C	1032710	HI0974.1	C	T
964472	*mutT*	C	T	1017375	*exoII*	C	A	1025846	*menB*	T	C	1034266	*panF*	T	C
965182	*kefC*	A	T	1017417	*exoII*	C	T	1025864	*menB*	T	C	1034377	*panF*	A	G
988537	HI0929	T	A	1017426	*exoII*	C	T	1025963	*menB*	C	T	1034380	*panF*	G	A
991317	*eno*	T	G	1017452	*exoII*	C	A	1025993	*menB*	G	C	1034404	*panF*	A	C
996089	HI0936	G	A	1017528	*exoII*	G	A	1026020	*menB*	G	T	1034872		C	T
1004762	*degS*	C	G	1017852	*exoII*	T	C	1026062	*menB*	C	T	1034883		G	A
1011540	*radC*	A	T	1017999	*exoII*	T	G	1026170	*menB*	T	G	1035622	*fic*	C	G
1011816	*radC*	G	A	1018547	HI0961	C	T	1026206	*menB*	G	A	1035678	*fic*	T	C
1012673	*dfp*	T	C	1018813	HI0961	T	A	1026209	*menB*	T	C	1035838	*fic*	T	C
1012677	*dfp*	C	A	1018857		A	G	1026230	*menB*	A	C	1035847	*fic*	C	A
1012708	*dfp*	T	C	1018993		C	T	1026479	*menC*	G	A	1035888	*fic*	G	T
1012709	*dfp*	T	G	1019077		A	G	1026580	*menC*	C	T	1036120	*prmA*	A	G
1012786	*dfp*	G	A	1019197		G	A	1026596	*menC*	A	C	1036291	*prmA*	C	T
1012810	*dfp*	C	A	1019203		C	T	1026656	*menC*	T	C	1036309	*prmA*	G	A
1012819	*dfp*	G	A	1019631		T	C	1026682	*menC*	A	T	1036549	*prmA*	T	A
1012966	*dfp*	T	C	1019875		A	C	1026877	*menC*	C	T	1036648	*prmA*	C	T
1012982	*dfp*	A	T	1019896		A	G	1026892	*menC*	A	G	1036687	*prmA*	T	C
1012993	*dfp*	T	A	1019926		G	A	1026895	*menC*	A	G	1036703	*prmA*	A	G
1013050	*dfp*	T	C	1019935		G	C	1027047	*menC*	T	C	1036759	*prmA*	C	T
1013092	*dfp*	C	T	1019989		C	T	1027098	*menC*	C	A	1036771	*prmA*	A	G
1013110	*dfp*	G	A	1020013		G	A	1027119	*menC*	T	C	1036783	*prmA*	T	C
1013113	*dfp*	T	C	1020023		A	G	1027207	*menC*	A	C	1036889	*prmA*	A	G
1013215	*dfp*	T	G	1020061		G	A	1027238	*menC*	C	G	1036999		T	C
1013225	*dfp*	G	T	1020064		A	G	1027276	*menC*	T	C	1037146	HI0979	G	A
1013348	*dut*	T	C	1020085		C	T	1027291	*menC*	T	A	1037759	HI0979	C	T
1013427	*dut*	T	A	1020109		G	A	1027354	*menC*	C	T	1037760	HI0979	A	G
1013451	*dut*	T	C	1020115		A	C	1027515	*aroQ*	C	T	1037918	HI0979	G	A
1013472	*dut*	C	T	1020244		C	G	1027707	*aroQ*	G	A	1038321	*fis*	T	C
1014132	*slmA*	A	G	1020313		A	G	1027995		T	C	1038596	*smpB*	G	A
1014483	*slmA*	C	A	1020355		A	G	1028154	*accB*	T	C	1038626	*smpB*	A	G
1015023	*crp*	G	T	1020679		T	C	1028244	*accB*	A	G	1038671	*smpB*	A	G
1015221	*crp*	C	T	1021583		T	A	1028341	*accB*	A	T	1038883	*smpB*	C	A
1015463		A	G	1021586		T	A	1028364	*accB*	G	A	1039256	*pfkA*	C	T
1015656		A	G	1023888	*mviN*	G	A	1028403	*accB*	G	C	1039437	*pfkA*	A	G
1015688		G	A	1024170	*mviN*	T	G	1028457	*accB*	T	A	1039443	*pfkA*	A	G
1016074	*rumB*	C	T	1024338		C	T	1029222	*accC*	C	T	1039455	*pfkA*	T	A
1016251	*rumB*	A	G	1024340		A	G	1029225	*accC*	C	T	1039458	*pfkA*	A	T
1016288	*rumB*	G	A	1024466	*rpsT*	T	C	1029237	*accC*	T	C	1039578	*pfkA*	A	G
1016337	*rumB*	C	T	1024756	*rpsT*	A	T	1029258	*accC*	A	C	1069382	HI1004	G	A
1016354	*rumB*	C	T	1024844		G	T	1029351	*accC*	C	G	1072736	*ispH*	A	T
1016366	*rumB*	A	G	1024992	HI0966	G	A	1029360	*accC*	C	T	1072819	*ispH*	G	T
1016444	*rumB*	T	C	1025024	HI0966	T	C	1029462	*accC*	T	C	1072888	*ispH*	G	A
1016516	*rumB*	T	C	1025048	HI0966	G	A	1029687	*accC*	T	C	1088553	*tktA*	C	T
1016673	*rumB*	G	A	1025062	HI0966	T	C	1032291		T	C	1091950	*lyx*	C	G
1016815	*rumB*	G	C	1025321	HI0966	T	C	1032313		A	G	1091951	*lyx*	G	C

*Position numbering based on that of NC_000907

Table A2.2d: List of polymorphisms in CA4 and CA6, part 4.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
1099040	*serB*	C	A	1392165	*nlpC*	A	T
1099698		A	T	1394221	HI1317	C	G
1100915	HI1036	A	T	1427066	*cdd*	C	A
1101939	HI1037	C	T	1429988	*putP*	T	A
1105881	HI1041	C	T	1431515	*cafA*	C	G
1108618	*napF*	C	G	1431516	*cafA*	G	C
1110933	*dmsB*	G	A	1432996	*glnS*	A	T
1114268	HI1048	C	G	1442596	*glgA*	T	G
1132239	*nrfB*	A	T	1447571	*pntA*	A	G
1132341	*nrfB*	A	T	1472592	*sbcB*	A	T
1135980	*hrpa*	A	T	1492796	HI1394	G	T
1139736	*cydB*	T	A	1493411		G	A
1142385		G	T	1493416		C	T
1161493	HI1099	C	T	1495274	*fumC*	T	C
1170104	*xylR*	C	T	1514945	HI1426	C	A
1172379	*nhaC*	A	T	1522710	*ybaK*	A	T
1183146	*comM*	A	T	1539782	*msrA*	C	T
1183188	*comM*	A	G	1544271	HI1462	G	A
1191409	*oppA*	T	C	1565250	HI1482	T	A
1191430	*oppA*	T	C	1569195		C	A
1201240	*murF*	A	G	1572018	HI1499	T	A
1202608	*mraY*	A	T	1592990		G	A
1205776	*ftsW*	A	T	1617029	HI1545	C	T
1236315	*ompA*	T	C	1618602	*aroG*	T	A
1236606	HI1165	C	T	1641556	*pykA*	C	T
1242825	*metX*	A	T	1659631	HI1590	A	C
1242945	*metX*	A	T	1659756	HI1590	C	G
1253815	*uvrD*	A	T	1659808	HI1590	A	C
1255853	*uvrD*	C	G	1663108		G	T
1255854	*uvrD*	G	C	1670319	HI1604	A	T
1260260	*gcvA*	T	A	1677620	*sfsA*	G	A
1278988	*lysS*	G	A	1693922		G	T
1279145		C	G	1696354	*purA*	A	T
1279146		G	C	1698672		G	T
1281204		C	A	1748745	HI1680	C	T
1287875	*lctP*	A	T	1765727	HI1695	A	T
1288052	*lctP*	C	A	1783777	*crr*	G	C
1302647	*aceF*	C	G	1783778	*crr*	C	G
1307119	*mgsA*	A	T	1823900	*murI*	T	A
1344453	*gyrA*	C	A	1828855		G	A
1360967	*infB*	G	A				

*Position numbering based on that of NC_000907

Table A2.3a: List of insertions and deletions in CA4 and CA6, part 1.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
32329	HI0030	G	GA	565145	*ureB*	T	TG
52066	HI0050m	C	CA	576723		GA	G
104315	*hitA*	A	AG	576860		C	CG
117269		G	GC	576862		T	TG
130905		AT	A	579246		G	GT
131983	*mltA*	GGT	G	579334	HI0559.1	A	AT
140552	*ppa*	TC	T	585570		GA	G
142122		TG	T	588149	*tex*	AG	A
143334	*afuB*	G	GCA	590522	*tex*	AT	A
145323		T	TTC	591067		TAA	T
162572	HI0147	C	CA	600488	*rpS7*	C	CT
162573	HI0147	C	CA	607968		G	GC
162605	HI0147	G	GA	608500	*pepE*	GC	G
166073		A	AT	615586		T	TG
170592		C	CT	615588		T	TG
173171	*fabD*	AG	A	615595		AG	A
173261		GC	G	620939	*ccrB*	G	GT
201555	*tatA*	AG	A	632170	*hemX*	CCT	C
201574	*tatA*	AG	A	647284		G	GA
204720		A	AG	647287		C	CAG
233416	*hsdS*	A	ACC	654433		GC	G
234640		A	AGG	663137	*fmt*	G	GC
235320		A	AC	678160		T	TG
240354		TC	T	678162		T	TG
263467		C	CT	721475	HI0680	G	GT
265439		TC	T	729849	*glpA*	GC	G
266933		G	GT	764934		C	CT
354945		G	GC	765075	*secE*	G	GT
369921		G	GC	787178		T	TAA
370788		GC	G	787337		A	AT
389958	HI0366	AC	A	787392		A	AT
391547	HI0367	AG	A	791739	HI0737	TC	T
393956	HI0370	G	GA	804315		C	CA
401967		AC	A	810715		AG	A
401979		TA	T	850970	*rpsM*	A	AG
415061		G	GA	853658		T	TA
428027	*accA*	AG	A	853846		A	AT
443488		C	CA	885804	*genX*	GT	G
443690		T	TG	892480		A	AG
446124		AT	A	916490		G	GA
467750		T	TA	921808		AG	A
485132	HI0463	G	GC	922034		CA	C
510227		AG	A	929031		CA	C
511410	*aphA*	CA	C	936290		G	GC
517873		CTCCGAT	C	951866		T	TG
534983		T	TCG	968666		G	GC
534996		T	TC	983710	*glyS*	C	CT
560060	*ureH*	GC	G	989395	HI0930	A	AT

*Position numbering based on that of NC_000907

Table A2.3b: List of insertions and deletions in CA4 and CA6, part 2.

Pos.*	Gene	Ref	CA	Pos.*	Gene	Ref	CA
997218	HI0938	TC	T	1347623		C	CA
1014632		AG	A	1370921	*truB*	A	AGC
1016976	*rumB*	A	AT	1377957		T	TG
1018743	HI0961	T	TA	1422174	*potC*	G	GT
1024796		GA	G	1439011	*glgX*	G	GC
1027982	*aroQ*	TA	T	1439050	*glgX*	G	GCC
1028638		G	GCC	1476596		TC	T
1034913		T	TA	1480065	*pstS*	G	GA
1036073	*prmA*	G	GA	1505885	HI1410	G	GC
1037040	HI0979	GC	G	1509059	HI1418	G	GC
1037047	HI0979	TA	T	1511667		A	AGC
1038089	HI0979	A	AG	1514364		A	AG
1066228	*trmE*	G	GC	1523231	*cspD*	CG	C
1068024	HI1004	GC	G	1523755		GC	G
1073492		GC	G	1525290		GT	G
1104427	*ureF*	C	CT	1527279	*ispA*	G	GCAC
1114929	*merT*	G	GC	1564858	*muB*	C	CG
1114934	*merT*	G	GC	1564863	*muB*	G	GC
1117514	HI1051	G	GA	1566212		T	TA
1120361		C	CCG	1568766		A	AG
1127714		G	GT	1569019		G	GC
1128978		T	TC	1570070	HI1493	T	TG
1128980		T	TC	1571863	HI1498.1	CG	C
1132302	*nrfB*	A	AACG	1575666	HI1502	T	TG
1139146		G	GC	1577703	HI1504	C	CG
1144510		CG	C	1580044		G	GC
1144973		AC	A	1587908		A	AG
1158617	*dsbE*	G	GA	1590314	HI1521	G	GC
1195317		TG	T	1604806	*grxA*	AG	A
1195324		TG	T	1607054		GT	G
1195695		C	CA	1608081		G	GC
1229388	HI1159m	TC	T	1617702	HI1546	CA	C
1231426	HI1162	G	GGC	1621283	*bioD*	G	GA
1231800	HI1162	T	TCCGC	1634585		TC	T
1239757		TC	T	1637524		G	GC
1243218		A	AG	1662092		A	AG
1243512	*sprT*	TG	T	1662266		AT	A
1249451		G	GC	1662939		AC	A
1257711		TA	T	1675969	*tyrS*	G	GT
1257768		TG	T	1687060		TG	T
1257775		TG	T	1689811	HI1625	AT	A
1257781		TG	T	1710521	*fbp*	G	GA
1277877	*lysS*	GA	G	1718873	*tldD*	A	AG
1296983	*uraA*	CA	C	1734148		G	GC
1307316		A	AG	1738448	HI1670	G	GC
1307904		T	TA	1775364	HI1703	ATC	A
1330978		AG	A	1782454	*ygiX*	G	GC
1331102		C	CT	1789588		GC	G
1331439		C	CT	1828820	*spoT*	A	AC
1341700		A	AC				

*Position numbering based on that of NC_000907

Table A2.4a: List of additional polymorphisms in CA4 only (region 1).

Pos.*	Gene	Ref	CA4	Pos.*	Gene	Ref	CA4	Pos.*	Gene	Ref	CA4
806546	*ndh*	A	T	812262	*tpx*	T	C	822626	*mutY*	G	A
806549	*ndh*	A	T	812332	*tpx*	C	T	822671	*mutY*	C	A
806585	*ndh*	C	G	812550		C	G	822854	*mutY*	A	G
806807	*ndh*	G	A	812599		A	G	822890	*mutY*	C	T
806834	*ndh*	G	A	812894	*purL*	G	A	822896	*mutY*	A	G
807719	HI0748	A	C	812996	*purL*	A	G	822962	*mutY*	T	A
807722	HI0748	A	G	813001	*purL*	T	C	822986	*mutY*	C	T
807907	HI0748	T	C	813011	*purL*	T	C	823052	*mutY*	A	G
808096	HI0748	C	T	813068	*purL*	T	G	823192	*mutY*	T	C
808378	HI0748	A	G	813107	*purL*	C	T	823254	*mutY*	A	G
808645	HI0748	C	T	813140	*purL*	T	C	823366	*mutY*	A	G
809428	HI0748	G	A	814550	*purL*	T	G	823429	*mutY*	A	G
809536	HI0748	C	T	814589	*purL*	C	T	823470	*mutY*	C	T
810686	*lexA*	T	C	814911	*purL*	T	C	823535	*mutY*	G	A
810778	*dapF*	T	G	814921	*purL*	T	C	823550	*mutY*	A	G
811315	*dapF*	G	C	814926	*purL*	A	T	823565	*mutY*	A	G
811318	*dapF*	T	C	815129	*purL*	T	G	823589	*mutY*	C	T
811327	*dapF*	T	C	815195	*purL*	G	C	823590	*mutY*	A	C
811393	*dapF*	T	C	815218	*purL*	T	A	823610	*mutY*	C	T
811410	*dapF*	G	A	815219	*purL*	A	C	823643	*mutY*	A	G
811511	*dapF*	T	G	815252	*purL*	C	T	823656	*mutY*	C	T
811531	*dapF*	C	A	815291	*purL*	T	A	823770	HI0760	T	C
811549	*dapF*	A	G	815328	*purL*	C	T	823782	HI0760	T	A
811993	*tpx*	C	T	815423	*purL*	C	T	823794	HI0760	C	G
812098	*tpx*	C	T	815429	*purL*	G	A	823831	HI0760	G	A
812116	*tpx*	A	G	815558	*purL*	A	G	823896	HI0760	G	A
812218	*tpx*	A	G	815699	*purL*	T	C	823965	HI0760	T	C

*Position numbering based on that of NC_000907

Table A2.4b: List of additional polymorphisms in CA4 only (region 2).

Pos.*	Gene	Ref	CA4
854888		T	C
854943		G	A
854978		G	T
855041		T	C
855531	HI0806	A	G

*Position numbering based on that of NC_000907

Table A2.4c: List of additional polymorphisms in CA4 only (region 3).

Pos.*	Gene	Ref	CA4	Pos.*	Gene	Ref	CA4
935574	HI0883	T	A	936817	*arcA*	G	A
935631	HI0883	G	A	936861	*arcA*	C	T
935689	HI0883	C	T	936862	*arcA*	A	G
935769	HI0883	A	C	936894	*arcA*	G	A
935901	HI0883	G	T	936904	*arcA*	G	A
935922	HI0883	A	G	936945	*arcA*	A	G
935940	HI0883	T	C	937027	*arcA*	C	T
935964	HI0883	G	A	937045	*arcA*	G	A
935967	HI0883	C	T	937132	*arcA*	C	T
936268		G	A	937183	*arcA*	A	G
936393		T	C	937218	*arcA*	T	C
936439		T	C	937272	*arcA*	T	G
936466		C	T	937364	*dipZ*	A	C
936503		C	A	937402	*dipZ*	T	C
936526	*arcA*	A	G	937451	*dipZ*	C	T
936577	*arcA*	G	T	937646	*dipZ*	A	G
936655	*arcA*	G	C	937733	*dipZ*	G	C
936757	*arcA*	T	C	937736	*dipZ*	T	A
936760	*arcA*	T	C	937769	*dipZ*	C	T
936790	*arcA*	G	A				

*Position numbering based on that of NC_000907

Table A2.5: List of additional insertions and deletions in CA4 only.

Pos.*	Gene	Ref	CA4
937308	*arcA*	TG	T
937841	*dipZ*	CG	C
947226	HI0893	G	GA

*Position numbering based on that of NC_000907

Table A2.6a: The *erm* BLAST score ratio output for genomes 1-14.*

	Isolate*													
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
erm(A)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.09	0.09	0.09	0.08
erm(F)	0.06	0.06	0	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.1	0.1	0.1	0.1	0.1	0.11	0.1	0.1	0.1	0.1	0.1	0.1	0.11	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.08	0.07	0.08	0.08	0.07	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.07	0.08
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.15
erm(V)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08
erm(W)	0.09	0.08	0.09	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.09	0.09	0.08	0.08
erm(X)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.13	0.12	0.12	0.12	0.12	0.13
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.15
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0	0	0	0.06	0	0	0	0.06	0	0	0
erm(36)	0.12	0.12	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.12	0.12	0.12	0.12	0.11
erm(37)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.07
erm(39)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.12	0.12	0.13	0.13	0.13	0.13	0.12
erm(40)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13
erm(41)	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6b: The *erm* BLAST score ratio output for genomes 15-28.*

	Isolate*													
Gene	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
erm(A)	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.09	0.08	0.09	0.09	0.08	0.09	0.09	0.09	0.09	0.09	0.09	0.08
erm(F)	0.06	0.06	0.06	0.06	0.06	0	0	0	0.06	0.06	0.06	0.06	0	0
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.11	0.1	0.11	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.11	0.12	0.11	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12	0.12	0.12	0.13
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.07	0.08	0.07	0.08	0.08	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.07	0.07
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.08	0.08	0.07	0.08	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.07
erm(W)	0.08	0.09	0.08	0.08	0.09	0.09	0.08	0.09	0.08	0.09	0.09	0.09	0.09	0.09
erm(X)	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.13	0.13
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0	0	0	0.06	0.06	0	0	0	0	0.06	0.06
erm(36)	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.11	0.11	0.12	0.12	0.12	0.12	0.12
erm(37)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(39)	0.13	0.13	0.13	0.12	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(40)	0.14	0.13	0.14	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(41)	0.11	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.12
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6c: The *erm* BLAST score ratio output for genomes 29-42.*

	Isolate*													
Gene	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
erm(A)	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.08	0.09	0.09	0.09	0.09	0.08	0.09	0.09	0.09	0.09	0.09	0.09
erm(F)	0.06	0.06	0	0	0.06	0.06	0.06	0	0.06	0.06	0.06	0.06	0.06	0.06
erm(G)	0.13	0.12	0.12	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.1	0.1	0.1	0.11	0.1	0.1	0.1	0.1	0.11	0.1	0.1	0.11	0.1	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.13	0.11	0.11	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.11	0.12	0.12
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.07	0.08	0.07	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.07	0.08	0.08
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08
erm(W)	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.09	0.09	0.09	0.08	0.09	0.09
erm(X)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.12	0.12
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0.06	0.06	0	0	0	0.06	0	0	0	0	0	0
erm(36)	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12	0.12
erm(37)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(39)	0.13	0.12	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(40)	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.14	0.14	0.13	0.13	0.14	0.13	0.13
erm(41)	0.12	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.15	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6d: The *erm* BLAST score ratio output for genomes 43-56.*

	Isolate*													
Gene	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
erm(A)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0.08	0.09	0.08
erm(F)	0.06	0.06	0.06	0.06	0.06	0.06	0	0.06	0.06	0.06	0	0	0.06	0.06
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.13	0.12	0.13	0.13	0.13
erm(H)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12	0.13	0.13	0.11	0.12	0.12	0.12
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.07	0.07	0.07	0.07	0.08	0.08
erm(T)	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.08	0.08	0.07	0.07	0.07	0.08	0.07
erm(W)	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.08	0.09	0.08	0.08	0.09	0.08
erm(X)	0.13	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.13	0.12	0.13	0.13	0.13
erm(Y)	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0	0	0	0.06	0	0	0.06	0.06	0.06	0.06	0
erm(36)	0.11	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.11	0.12	0.12	0.12	0.12	0.11
erm(37)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.07	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.07
erm(39)	0.12	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.12
erm(40)	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.14	0.13	0.13	0.13
erm(41)	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.12	0.11	0.12	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6e: The *erm* BLAST score ratio output for genomes 57-70.*

	Isolate*													
Gene	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**
erm(A)	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.13	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(F)	0.06	0.06	0.06	0.06	0.06	0	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.1	0.1	0.11	0.1	0.1	0.11	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.12	0.11	0.12	0.12	0.13	0.11	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.12
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.08	0.07	0.08	0.07	0.07	0.07	0.08	0.07	0.08	0.07	0.07	0.07	0.08	0.07
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(W)	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.08	0.09	0.08	0.08	0.08	0.09	0.08
erm(X)	0.12	0.13	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0.06	0	0	0	0.06	0	0	0.06	0	0	0
erm(36)	0.12	0.12	0.12	0.11	0.11	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12
erm(37)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08
erm(39)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(40)	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.14	0.13	0.13
erm(41)	0.11	0.12	0.11	0.11	0.12	0.11	0.11	0.12	0.11	0.12	0.12	0.11	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6f: The *erm* BLAST score ratio output for genomes 71-81.*

	Isolate*										
Gene	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**
erm(A)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.08	0.09
erm(F)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.1	0.11	0.1	0.11	0.1	0.1	0.1	0.1	0.1	0.1	0.1
erm(N)	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.08	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08
erm(W)	0.09	0.09	0.09	0.08	0.09	0.09	0.09	0.09	0.09	0.08	0.09
erm(X)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.12
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0.06	0	0	0	0	0	0	0
erm(36)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12
erm(37)	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08
erm(39)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.12	0.13
erm(40)	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(41)	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.6g: The *erm* BLAST score ratio output for genomes 82-92.*

	Isolate*										
Gene	**82**	**83**	**84**	**85**	**86**	**87**	**88**	**89**	**90**	**91**	**92**
erm(A)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14
erm(B)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(C)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(D)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(E)	0.09	0.09	0.09	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.09
erm(F)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0	0.06	0
erm(G)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(H)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.11	0.1	0.11
erm(N)	0	0	0	0	0	0	0	0	0	0	0
erm(O)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
erm(Q)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.11
erm(R)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
erm(S)	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07
erm(T)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(V)	0.08	0.08	0.08	0.07	0.07	0.08	0.08	0.08	0.08	0.07	0.08
erm(W)	0.09	0.09	0.09	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08
erm(X)	0.12	0.12	0.12	0.13	0.13	0.12	0.12	0.12	0.12	0.13	0.12
erm(Y)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
erm(31)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
erm(32)	0	0	0	0	0	0	0	0	0	0	0
erm(33)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
erm(34)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
erm(35)	0	0	0	0	0	0.06	0	0	0.06	0	0
erm(36)	0.11	0.12	0.12	0.11	0.11	0.11	0.12	0.11	0.12	0.11	0.12
erm(37)	0	0	0	0	0	0	0	0	0	0	0
erm(38)	0.08	0.08	0.08	0.07	0.07	0.07	0.08	0.08	0.08	0.07	0.08
erm(39)	0.12	0.13	0.13	0.12	0.12	0.13	0.13	0.12	0.13	0.12	0.13
erm(40)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.14
erm(41)	0.12	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
erm(42)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
erm(43)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7a: The efflux gene BLAST score ratio output for genomes 1-14.*

	Isolate*													
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.16	0.19	0.19
lsa(C)	0.15	0.15	0.14	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
lsa(E)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14
mef(A)	0.06	0.06	0.05	0.05	0.05	0.05	0.06	0.05	0.06	0.05	0.06	0.05	0.05	0.06
mef(E)	0.05	0	0	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
msr(A)	0.15	0.15	0.16	0.15	0.16	0.16	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.15
msr(C)	0.19	0.2	0.17	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.17	0.19	0.19
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.18	0.17	0.16	0.17	0.17	0.17	0.18	0.17	0.17	0.16	0.17	0.16	0.18	0.17
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.21	0.2	0.21	0.21	0.21	0.21	0.21	0.21	0.2	0.21	0.2	0.21	0.21
tlr(C)	0.21	0.21	0.21	0.2	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.19	0.17	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.17	0.19	0.19
vga(B)	0.19	0.19	0.17	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.17	0.19	0.19
vga(C)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
vga(D)	0.17	0.16	0.17	0.17	0.16	0.16	0.16	0.17	0.16	0.16	0.17	0.16	0.17	0.17
vga(E)	0.19	0.2	0.19	0.19	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7b: The efflux gene BLAST score ratio output for genomes 15-28.*

	Isolate*													
Gene	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.15	0.14	0.15	0.15	0.15	0.15	0.15	0.15	0.14	0.15	0.15	0.15	0.15	0.15
lsa(E)	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
mef(A)	0.05	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.06	0.06
mef(E)	0.05	0.05	0.05	0.05	0.05	0	0	0	0.05	0.05	0.05	0	0	0.05
msr(A)	0.15	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.15	0.15	0.16	0.16	0.15
msr(C)	0.19	0.19	0.19	0.19	0.19	0.2	0.19	0.2	0.19	0.17	0.19	0.2	0.2	0.19
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.18	0.17	0.17	0.18	0.18	0.17	0.17	0.17	0.17	0.16	0.18	0.17	0.17	0.17
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
vga(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.19	0.19	0.19
vga(C)	0.18	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.18	0.18	0.18	0.18
vga(D)	0.17	0.16	0.16	0.16	0.17	0.16	0.17	0.17	0.16	0.17	0.16	0.16	0.16	0.17
vga(E)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.2	0.19	0.2	0.2	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7c: The efflux gene BLAST score ratio output for genomes 29-42.*

	Isolate*													
Gene	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.14	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14	0.15	0.15	0.15	0.15
lsa(E)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
mef(A)	0.04	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.05	0.06	0.05	0.05	0.06	0.06
mef(E)	0	0	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
msr(A)	0.15	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.15	0.16	0.15	0.16	0.15
msr(C)	0.19	0.19	0.19	0.2	0.19	0.19	0.2	0.19	0.19	0.15	0.19	0.19	0.19	0.19
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.17	0.17	0.17	0.16	0.17	0.17	0.17	0.17	0.17	0.16	0.18	0.18	0.17	0.17
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.19	0.19	0.19
vga(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19	0.19	0.19	0.19
vga(C)	0.18	0.19	0.18	0.18	0.18	0.18	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.18
vga(D)	0.16	0.17	0.17	0.16	0.17	0.16	0.17	0.17	0.16	0.16	0.17	0.16	0.16	0.16
vga(E)	0.19	0.19	0.19	0.19	0.19	0.19	0.2	0.19	0.2	0.19	0.19	0.19	0.19	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7d: The efflux gene BLAST score ratio output for genomes 43-56.*

	Isolate*													
Gene	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.19	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
lsa(E)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
mef(A)	0.06	0.05	0.06	0.05	0.06	0.05	0.06	0.05	0.04	0.06	0.06	0.06	0.06	0.06
mef(E)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
msr(A)	0.15	0.15	0.15	0.15	0.15	0.15	0.16	0.15	0.15	0.15	0.16	0.16	0.15	0.16
msr(C)	0.19	0.17	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.2	0.19	0.19
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.17	0.16	0.17	0.17	0.18	0.17	0.17	0.18	0.17	0.18	0.17	0.17	0.18	0.18
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.2	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.17	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
vga(B)	0.19	0.17	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
vga(C)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
vga(D)	0.17	0.16	0.16	0.17	0.16	0.17	0.17	0.16	0.16	0.16	0.17	0.17	0.16	0.16
vga(E)	0.19	0.19	0.19	0.19	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7e: The efflux gene BLAST score ratio output for genomes 57-70.*

	Isolate*													
Gene	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14	0.15
lsa(E)	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
mef(A)	0.05	0.04	0.06	0.06	0.04	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.06	0.06
mef(E)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0
msr(A)	0.15	0.15	0.15	0.16	0.15	0.15	0.16	0.16	0.16	0.15	0.16	0.16	0.15	0.16
msr(C)	0.19	0.2	0.19	0.2	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.15	0.2
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.18	0.17	0.18	0.17	0.16	0.17
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19
vga(B)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.17	0.19
vga(C)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.18
vga(D)	0.16	0.17	0.17	0.16	0.16	0.17	0.17	0.16	0.17	0.16	0.16	0.16	0.16	0.16
vga(E)	0.19	0.2	0.2	0.19	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.2

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7f: The efflux gene BLAST score ratio output for genomes 71-81.*

	Isolate*										
Gene	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**
car(A)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.15	0.15	0.15	0.15	0.15	0.15	0.16	0.14	0.15	0.14	0.15
lsa(E)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
mef(A)	0.06	0.06	0.05	0.06	0.05	0.06	0.05	0.06	0.04	0.04	0.06
mef(E)	0.05	0.05	0.05	0.05	0.05	0	0.05	0.05	0.05	0.05	0.05
msr(A)	0.15	0.15	0.15	0.15	0.15	0.16	0.16	0.15	0.15	0.15	0.15
msr(C)	0.19	0.19	0.19	0.2	0.19	0.19	0.2	0.2	0.19	0.19	0.19
msr(D)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.18	0.19
msr(E)	0.17	0.17	0.17	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.18
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.19	0.2
srm(B)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.2	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
vga(B)	0.19	0.19	0.19	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19
vga(C)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.18
vga(D)	0.17	0.17	0.16	0.17	0.17	0.17	0.16	0.17	0.17	0.16	0.16
vga(E)	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.7g: The efflux gene BLAST score ratio output for genomes 82-92.*

	Isolate*										
Gene	**82**	**83**	**84**	**85**	**86**	**87**	**88**	**89**	**90**	**91**	**92**
car(A)	0.21	0.21	0.21	0.2	0.21	0.21	0.21	0.21	0.21	0.21	0.21
lmr(A)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
lsa(B)	0.16	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
lsa(C)	0.14	0.15	0.14	0.15	0.14	0.15	0.16	0.15	0.14	0.14	0.15
lsa(E)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15
mef(A)	0.05	0.06	0.06	0.04	0.04	0.06	0.06	0.05	0.06	0.06	0.05
mef(E)	0	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
msr(A)	0.15	0.15	0.16	0.15	0.16	0.15	0.16	0.16	0.15	0.15	0.15
msr(C)	0.17	0.2	0.15	0.19	0.17	0.19	0.19	0.19	0.17	0.17	0.19
msr(D)	0.19	0.19	0.19	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19
msr(E)	0.16	0.17	0.16	0.17	0.16	0.18	0.17	0.17	0.16	0.16	0.18
ole(B)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
ole(C)	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
sal(A)	0.2	0.2	0.2	0.19	0.2	0.2	0.2	0.2	0.2	0.2	0.2
srm(B)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.2	0.2	0.21
tlr(C)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
vga(A)	0.17	0.19	0.17	0.19	0.17	0.19	0.19	0.19	0.19	0.19	0.19
vga(B)	0.17	0.19	0.17	0.19	0.18	0.19	0.19	0.19	0.18	0.18	0.19
vga(C)	0.18	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.19	0.19	0.18
vga(D)	0.16	0.17	0.16	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.17
vga(E)	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8a: The deactivating gene BLAST score ratio output for genomes 1-14.*

	Isolate*													
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
ere(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(A)	0	0	0.09	0.09	0	0	0	0	0	0.09	0	0.09	0	0
lnu(B)	0	0.06	0	0	0	0	0	0	0	0	0	0	0	0
lnu(C)	0	0	0.08	0.08	0	0	0	0	0	0.08	0	0.08	0	0
lnu(D)	0	0.09	0.1	0.1	0	0	0	0	0	0.1	0	0.1	0	0
lnu(E)	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.09
lnu(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0.05	0	0	0	0	0	0	0.05	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.09	0.07	0.07	0.09	0.09	0.09	0.07	0.07	0.09	0.07	0.09	0.07	0.09	0.09
vat(D)	0.1	0.09	0	0.09	0.09	0.1	0.09	0.09	0.09	0	0.09	0	0.09	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8b: The deactivating gene BLAST score ratio output for genomes 15-28.*

	Isolate*													
Gene	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
ere(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0	0	0	0	0	0	0	0	0	0.04	0
lnu(A)	0	0	0	0	0	0	0	0	0	0	0	0.09	0	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0	0.06	0	0
lnu(C)	0	0	0	0	0	0	0	0	0	0	0	0.08	0	0
lnu(D)	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0
lnu(E)	0.08	0.08	0.08	0.09	0.08	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0	0.09
lnu(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.09	0.09	0.07	0.09	0.09	0.09	0.09	0.07	0.07	0.07	0.07	0.07	0.07	0.07
vat(D)	0.09	0.1	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8c: The deactivating gene BLAST score ratio output for genomes 29-42.*

	Isolate*													
Gene	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
ere(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0.04	0	0	0	0	0	0	0	0	0	0
lnu(A)	0.09	0.09	0	0	0	0.09	0.09	0	0	0	0	0	0	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(C)	0.08	0.08	0	0	0	0.08	0.08	0	0	0	0	0	0	0
lnu(D)	0.1	0.1	0	0	0	0.1	0.1	0	0	0	0	0	0	0
lnu(E)	0.09	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.09
lnu(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0.06	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.09	0.09	0.09	0.07	0.09	0.07	0.07	0.09	0.09	0.09	0.07	0.07	0.07	0.09
vat(D)	0.09	0.09	0.09	0.09	0.09	0	0.09	0.09	0.1	0.09	0.09	0.09	0	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8d: The deactivating gene BLAST score ratio output for genomes 43-56.*

	Isolate*													
Gene	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
ere(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(A)	0	0.09	0	0	0	0	0	0	0.09	0	0	0	0	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(C)	0	0.08	0	0	0	0	0	0	0.08	0	0	0	0	0
lnu(D)	0	0.1	0	0	0	0	0	0	0.1	0	0	0	0	0
lnu(E)	0.09	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0	0.08	0.09
lnu(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.09	0.07	0.09	0.07	0.09	0.07	0.09	0.09	0.09	0.07	0.09	0.07	0.07	0.09
vat(D)	0.09	0	0.09	0	0.09	0	0.09	0.1	0.09	0.09	0.09	0.09	0.09	0.09
vat(E)	0.1	0.1	0.1	0.09	0.1	0.09	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8e: The deactivating gene BLAST score ratio output for genomes 57-70.*

	Isolate*													
Gene	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**
ere(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0.04	0	0	0	0	0	0	0	0	0	0
lnu(A)	0	0.09	0	0	0.09	0	0	0	0	0	0	0.09	0	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0.06
lnu(C)	0	0.08	0	0	0.08	0	0	0	0	0	0	0.08	0	0
lnu(D)	0	0.1	0	0	0.1	0	0	0	0	0	0	0.1	0	0.09
lnu(E)	0.09	0.09	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.09	0.08	0.09	0.08	0.08
lnu(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0.05	0	0	0	0	0.05	0	0	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0.06	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.07	0.09	0.09	0.07	0.09	0.07	0.09	0.09	0.07	0.07	0.09	0.07	0.09	0.07
vat(D)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0.05	0	0	0	0	0	0.05	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

Table A2.8f: The deactivating gene BLAST score ratio output for genomes 71-81.*

	Isolate*										
Gene	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**
ere(A)	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0	0	0	0	0.04	0	0	0
lnu(A)	0.09	0	0	0	0.09	0	0.09	0	0.09	0.09	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0
lnu(C)	0.08	0	0	0	0.08	0	0.08	0	0.08	0.08	0
lnu(D)	0.1	0	0.09	0	0.1	0.09	0.1	0	0.1	0.1	0
lnu(E)	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.09	0.08	0.09	0.08
lnu(F)	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0.05	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0	0	0	0	0	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.07	0.09	0.07	0.07	0.07	0.09	0.09	0.07	0.09	0.09	0.07
vat(D)	0.09	0.09	0.09	0.09	0	0.09	0.09	0.09	0.09	0.09	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0

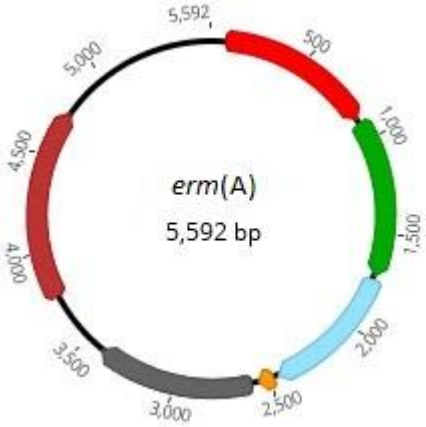
*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

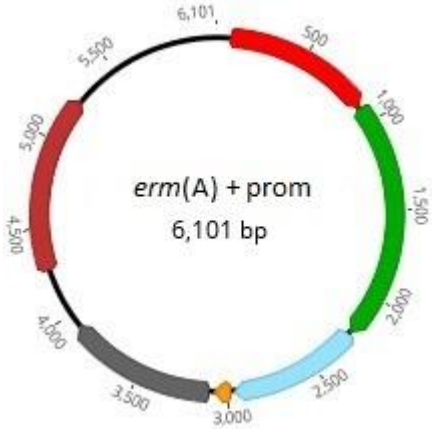
Table A2.8g: The deactivating gene BLAST score ratio output for genomes 82-92.*

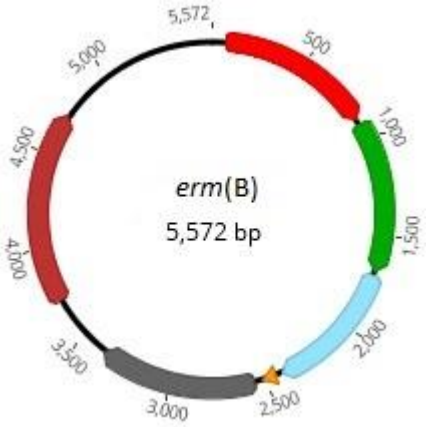
	Isolate*										
Gene	**82**	**83**	**84**	**85**	**86**	**87**	**88**	**89**	**90**	**91**	**92**
ere(A)	0	0	0	0	0	0	0	0	0	0	0
ere(B)	0	0	0	0	0	0	0.04	0	0	0	0
lnu(A)	0.09	0.09	0	0.09	0.09	0	0	0.09	0.09	0.09	0
lnu(B)	0	0	0	0	0	0	0	0	0	0	0
lnu(C)	0.08	0.08	0	0.08	0.08	0	0	0.08	0.08	0.08	0
lnu(D)	0.1	0.1	0	0.1	0.1	0	0	0.1	0.1	0.1	0
lnu(E)	0.08	0.09	0.08	0.09	0.09	0.08	0.09	0.09	0.09	0.09	0.08
lnu(F)	0	0	0	0	0	0	0	0	0	0	0
mph(A)	0	0	0	0	0	0	0	0	0	0	0
mph(B)	0	0	0	0	0	0	0	0	0	0	0
mph(C)	0	0	0	0	0	0	0	0	0	0	0
mph(E)	0	0	0	0	0	0	0	0	0	0	0
mph(F)	0	0	0	0	0	0	0	0	0	0	0
vat(A)	0	0	0	0	0	0	0	0	0	0	0
vat(B)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
vat(C)	0.09	0.07	0.09	0.09	0.09	0.07	0.07	0.07	0.07	0.07	0.09
vat(D)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0	0	0	0.09
vat(E)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
vgb(A)	0	0	0	0	0	0	0	0	0	0	0
vgb(B)	0	0	0	0	0	0	0	0	0	0	0

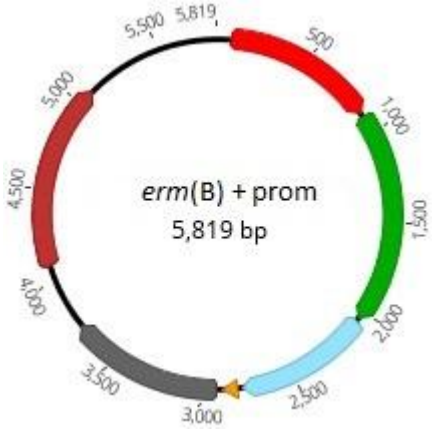
*Refer to Table 1 in Chapter 5 for interpretation of isolate numbers

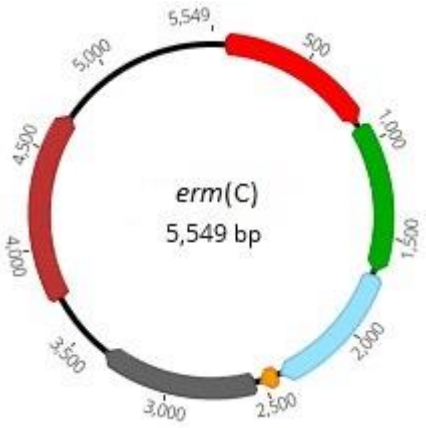
Appendix 3: Constructed plasmids used for cloning

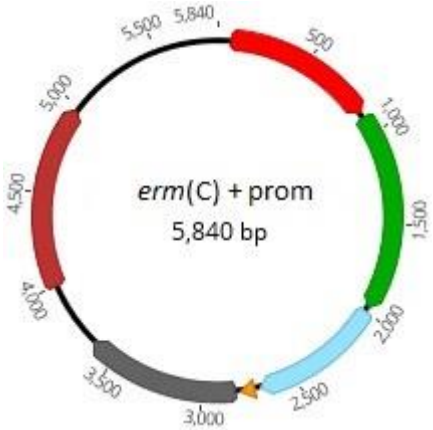


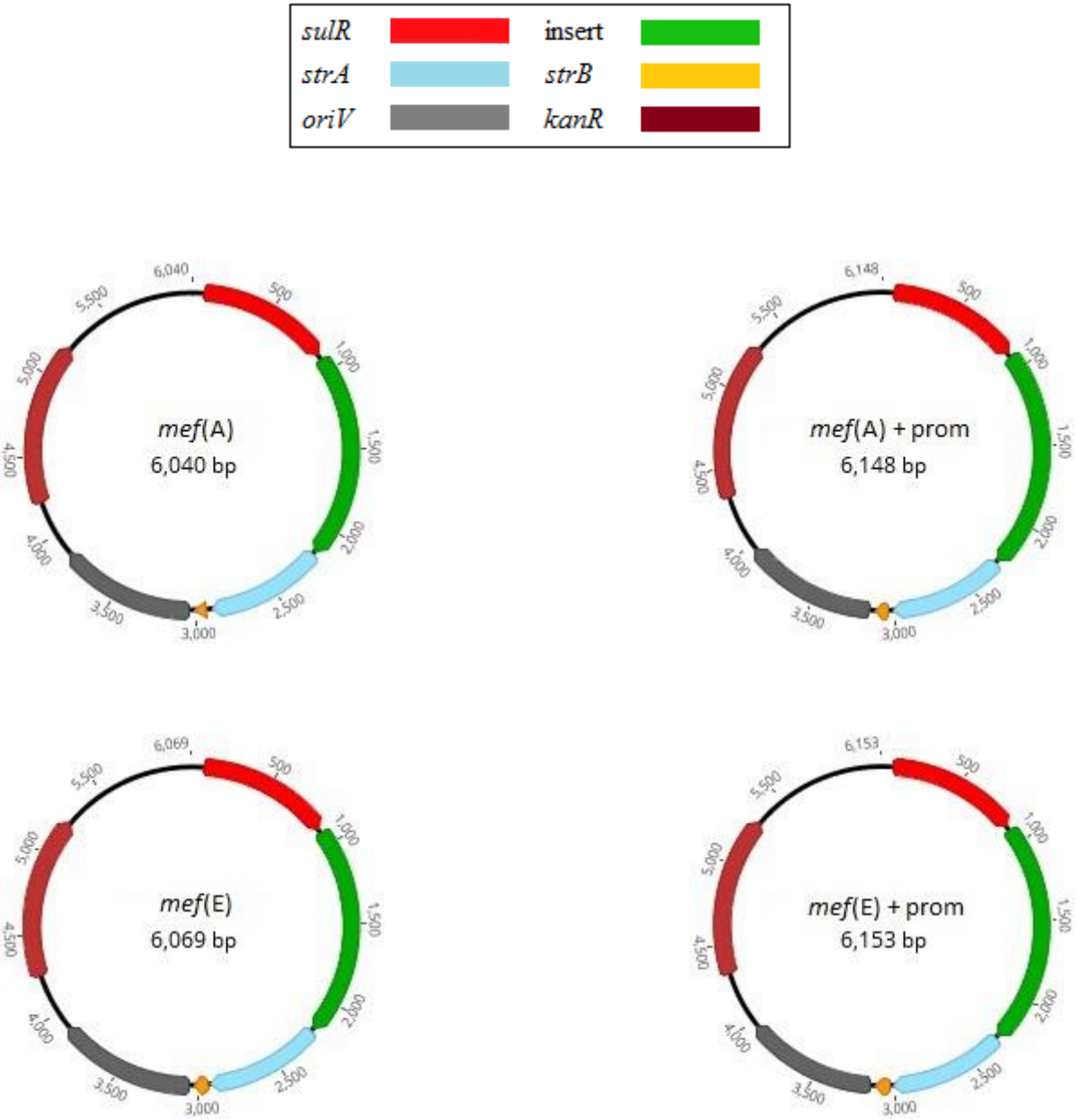











sulR
insert
strA
strB
oriV
kanR
mef(A)
6,040 bp
mef(A) + prom
6,148 bp
mef(E)
6,069 bp
mef(E) + prom
6,153 bp